REMARKS

Claims 26, 28-31, and 44-59 were previously pending and under examination.

Claim 26 has been amended herein. Support for the amendment can be found throughout the specification and claims as filed. More particularly, support can be found, *e.g.*, in paragraphs [0040], [0166], and [0171] of the specification as published (US 2008/0058431). No new matter has been added by the amendments.

Claims 46-52 have been cancelled without prejudice. Applicants submit that the cancellation of a claim makes no admission as to its patentability and reserve the right to pursue the subject matter of the cancelled claim in this or any other patent application.

Rejections under 35 U.S.C. § 112, First Paragraph – Enablement

The Examiner has maintained the rejection of Claims 26, 28-31, and 44-52 under 35 U.S.C. § 112, first paragraph, as allegedly failing to provide enablement for the full scope of the claims. The Examiner cites to Grossman *et al.* (PNAS, 2009, 106:16704-16709), Nakamura *et al.* (PNAS, 2009, 106:11270-11275), and Saxton *et al.* (1997, EMBO J, 16:2352-2364) as teaching conditional knockouts of Shp2 that lead to phenotypes other than those in the present claims, and reiterates the assertion that Reece (Analysis of Genes and Genomes) teaches that CamK2a "drives expression only in the neurons of hippocampus." *See Office Action* at pp. 2-3. The Examiner also asserts that the claims encompass a mouse "wherein there only needs to [be] a single cell that does not comprise the genetic modification." *See Office Action* at pp. 3-4.

Without acquiescing to the pending rejections, and solely to advance prosecution, Applicants have amended Claim 1 to clarify that the genome of the claimed genetically modified mouse "comprises a homozygous disruption of the endogenous Shp2 gene in only neuronal forebrain cells of the mouse."

As discussed in Applicants' response of October 24, 2011, the references cited by the Examiner disclose conditional mutations of Shp2 using the Wnt1-cre promoter, which introduces recombination in neural crest cells (NCCs). *See Grossman* at p. 16704. These NCCs are migratory cells that give rise to melanocytes, connective tissue of the head, and glial and neuronal cells of the peripheral nervous system, but do not form any part of the forebrain. Similarly,

conditional mutation of Shp2 in myelinating Schwann cells resulted in reduced myelination of peripheral nerves, but again this is unrelated to disruption of forebrain cells. *See Nakamura* at Abstract; *Grossman* at pp. 16705-16706. And although non-conditional mutations of Shp2 in embryonic stem cells resulted in embryonic lethality, this again is unrelated to a knockout in forebrain cells.

Because none of these references relate to performing a knockout of Shp2 genes in forebrain cells, one of ordinary skill in the art at the time of filing would be fully enabled for using a forebrain specific promoter to perform a knockout in the forebrain because they would not have looked to these unrelated references to note that such a knockout may not function properly, or cause abnormal phenotypes.

The Examiner also argued that according to the Reece reference, the CamK2a promoter was limited to expression in the hippocampus, and not expressed in other areas of the forebrain. Applicants disagree. As discussed in Applicants' response of April 15, 2011, the Reece reference mischaracterizes the Mayford reference it relies on to support the assertion that the CamK2a promoter drives expression only in the neurons of the hippocampus. In particular, Reece states that:

> ...the promoter of the calcium-calmodulin dependent kinase II (CaMKIIα) gene drives expression only in the neurons of the hippocampus (Mayford *et al.*, 1996). Such an approach works well, provided that a suitable tissue-specific promoter is available (Table 13.1).

Reece at p. 389 (emphases added). However, the Mayford *et al.* 1996 article (attached as Exhibit 1) cited in this passage actually teaches that "CaMKIIα is a serine-threonine protein kinase that is restricted to the forebrain (12-14). It is expressed in the neurons of the neocortex, the hippocampus, the amygdala, and the basal ganglia." *Mayford et al.* 1996, at p. 1678. The Mayford *et al.* 1996 article also teaches that the CAMKII promoter "can limit expression to forebrain neurons generally," and provides an example of a transgenic mouse with a CAMKIIα promoter that demonstrates "uniform [expression of a transgene of interest] throughout the forebrain, neocortex, hippocampus, amygdala, and striatum." *Id.*, at p. 1679.

Applicants therefore respectfully submit that the full scope of the amended claims is enabled in light of the teachings of the Specification and the knowledge in the art at the time of

filing of the instant application. Applicants therefore respectfully request that the Examiner withdraw the rejection of Claim 26 and claims dependent therefrom (*i.e.*, Claims 28- 31, 44, and 45) under 35 U.S.C. § 112, first paragraph.

No Disclaimers or Disavowals

Although the present communication may include alterations to the application or claims, or characterizations of claim scope or referenced art, Applicants are not conceding in this application that previously pending claims are not patentable over the cited references. Rather, any alterations or characterizations are being made to facilitate expeditious prosecution of this application. Applicants reserve the right to pursue at a later date any previously pending or other broader or narrower claims that capture any subject matter supported by the present disclosure, including subject matter found to be specifically disclaimed herein or by any prior prosecution. Accordingly, reviewers of this or any parent, child or related prosecution history shall not reasonably infer that Applicants have made any disclaimers or disavowals of any subject matter supported by the present application.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: March 19, 2012

By:/Michael L Fuller/
Michael L. Fuller
Registration No. 36,516
Attorney of Record
Customer No. 20995
(858) 707-4000

12951245
031612

EXHIBIT 1

Control of Memory Formation Through Regulated Expression of a CaMKII Transgene

Mark Mayford, Mary Elizabeth Bach, Yan-You Huang, Lei Wang, Robert D. Hawkins, Eric R. Kandel*

One of the major limitations in the use of genetically modified mice for studying cognitive functions is the lack of regional and temporal control of gene function. To overcome these limitations, a forebrain-specific promoter was combined with the tetracycline transactivator system to achieve both regional and temporal control of transgene expression. Expression of an activated calcium-independent form of calcium-calmodulin–dependent kinase II (CaMKII) resulted in a loss of hippocampal long-term potentiation in response to 10-hertz stimulation and a deficit in spatial memory, a form of explicit memory. Suppression of transgene expression reversed both the physiological and the memory deficit. When the transgene was expressed at high levels in the lateral amygdala and the striatum but not other forebrain structures, there was a deficit in fear conditioning, an implicit memory task, that also was reversible. Thus, the CaMKII signaling pathway is critical for both explicit and implicit memory storage, in a manner that is independent of its potential role in development.

Explicit memory—a memory for facts, places, and events—requires the hippocampus and related medial temporal lobe structures (*1*), whereas implicit memory—a memory for perceptual and motor skills—involves a variety of anatomical systems (*2*). For example, one form of implicit memory, that for conditioned fear, involves the amygdala (*3*).

Studies with genetically modified animals have sought to relate specific genes to specific forms of explicit or implicit memory storage (*4–8*). However, current methodology does not allow one to distinguish between a direct effect on memory or its underlying synaptic mechanisms and an indirect effect on the development of the neuronal circuits in which the memory storage occurs (*4, 9*). In addition, the gene under study is typically overexpressed or ablated throughout the entire brain. As a result, the genetic modifications often affect, indiscriminately, both implicit and explicit memory as well as perceptual or motor performance. Thus, to analyze the molecular contribution of a given gene to a particular type of memory, it is essential not only to control the timing of expression but also to restrict expression to appropriate cell populations.

To address these issues and to achieve regulated transgene expression in restricted regions of the forebrain, we used a forebrain-specific promoter in combination with the tetracycline transactivator (tTA) developed by Bujard and his colleagues (*10, 11*). We examined the role of CaMKII signaling in synaptic plasticity as well as in implicit and explicit memory storage.

CaMKIIα is a serine-threonine protein kinase that is restricted to the forebrain (*12–14*). It is expressed in the neurons of the neocortex, the hippocampus, the amygdala, and the basal ganglia. After a brief exposure to Ca^{2+}, CaMKII can convert to a Ca^{2+}-independent state through an autophosphorylation at Thr^{286} (*12, 14–17*). This ability to become persistently active in response to a transient Ca^{2+} stimulus led to the suggestion that CaMKII may be a molecular substrate of memory (*18*). Targeted disruption of the CaMKIIα gene produces deficits in long-term potentiation (LTP) and severely impairs performance on hippocampal-dependent memory tasks (*5, 6*). Mutation of Thr^{286} to Asp in CaMKIIα mimics the effect of autophosphorylation at Thr^{286} and converts the enzyme to a Ca^{2+}-independent form (*15, 17*). Transgenic expression of this dominant mutation of CaMKIIα (CaMKII-Asp^{286}) results in a systematic shift in response to low-frequency stimulation such that long-term depression (LTD) is favored in the transgenic mice (*7*). Thus, although Schaffer collateral LTP in response to 100-Hz tetanus is not altered, LTP is eliminated in the range of 5 to 10 Hz, a frequency (the theta frequency) characteristic of the endogenous oscillation in neuronal activity seen in the hippocampus of animals during spatial exploration (*19*). Correlated with this selective deficit in LTP in the theta frequency range is a severe defect in spatial memory (*8*). We now have reexamined these phenomena with regulated expression of the CaMKII-Asp^{286} transgene.

Doxycycline regulation of transgene expression. The first type of mouse we generated to achieve regulated expression of CaMKII-Asp^{286} in forebrain neurons (Fig. 1A) expressed the tTA gene under the control of the CaMKIIα promoter (line B), which limits expression of the tTA transgene to neurons of the forebrain (*20*). In the second type of mouse, the tTA-responsive tet-O promoter is linked to the target gene of interest, in this case either *lacZ* or the CaMKII-Asp^{286} gene. The tTA gene expresses a eukaryotic transcription activator that binds to and activates transcription

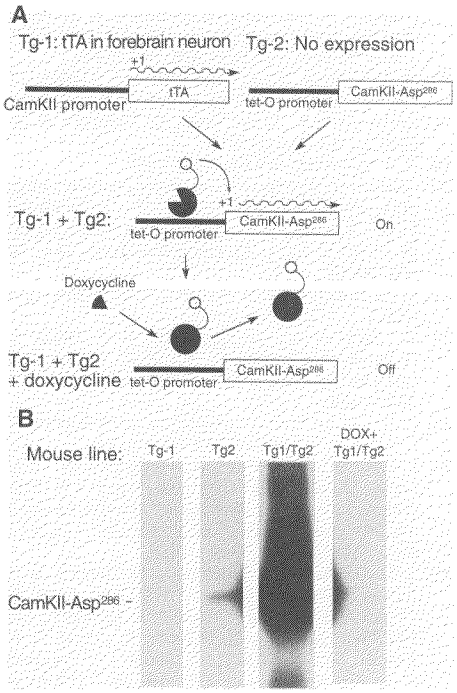

Fig. 1. Regulation of the CaMKII-Asp^{286} transgene with the tTA system. (**A**) Strategy used to obtain forebrain-specific doxycycline-regulated transgene expression. Two independent lines of transgenic mice are obtained, and the two transgenes are introduced into a single mouse through mating. (**B**) Quantitation by RT-PCR Southern blot of CaMKII-Asp^{286} expression from the tet-O promoter. RT-PCR was performed on total forebrain RNA and probed for expression of the CaMKII-Asp^{286} mutant mRNA as described (*7, 21*). Tg1, mouse carrying only the CaMKII promoter–tTA transgene (line B). Tg2, mouse carrying only the tet-O–CaMKII-Asp^{286} transgene (line 21). Tg1/Tg2, double transgenic mouse carrying both the CaMKII promoter-tTA transgene (line B) and tet-O–CaMKII-Asp^{286} (line 21) transgenes. Tg1/Tg2 + Dox, double transgenic mouse treated with doxycycline (2 mg/ml) plus 5% sucrose in the drinking water for 4 weeks.

The authors are at the Center for Neurobiology and Behavior, College of Physicians and Surgeons of Columbia University, and Howard Hughes Medical Institute, 722 West 168 Street, New York, NY 10032, USA.

*To whom correspondence should be addressed.

from the tet-O promoter element; this transcription is blocked by the tetracycline analog doxycycline (*10*). When both the tet-O and tTA transgenes were introduced into the same mouse, the tet-O–linked gene was activated, but only in those cells that express tTA.

We assessed the regulation of the CaMKII-Asp^{286} transgene using a reverse transcriptase–polymerase chain reaction (RT-PCR) Southern (DNA) blot (*21*) to detect only the mutant transcripts (Fig. 1B). Mice carrying either one of the transgenes alone show little or no expression of CaMKII-Asp^{286} mRNA. When both transgenes were introduced into the same mouse, there was a large activation of CaMKII-Asp^{286} expression. The expression of this transgene was completely suppressed when the mice were given doxycycline (2 mg/ml) in the drinking water for 4 weeks.

Restricted expression of the tet-O–linked transgenes. We examined the expression of β-galactosidase in two tet-O *lacZ* reporter lines of mice that also carried the CaMKIIα promoter–tTA transgene (Fig. 2A). In the first line, expression was uniform throughout the forebrain, neocortex, hippocampus, amygdala, and striatum. This pattern mimics the expression of the endogenous CaMKIIα gene (*13*). In the second *lacZ* line, expression was observed throughout the forebrain, but surprisingly, expression was absent in the CA3 pyramidal cell body layer of the hippocampus (Fig. 2B).

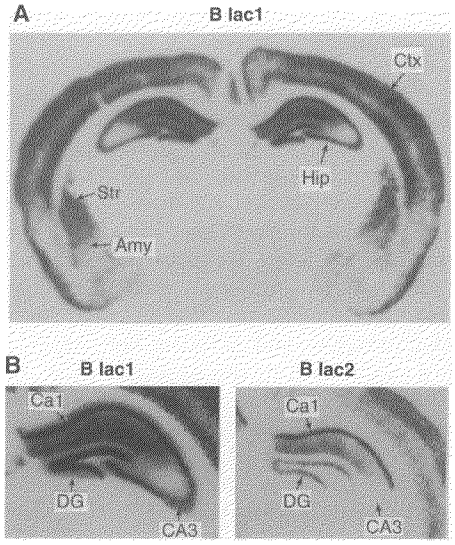

Fig. 2. Forebrain-specific activation of a tet-O–*lacZ* transgene. (**A**) Coronal section of double transgenic line B lacI stained with X-Gal as described (*42*). Ctx, cerebral cortex; Str, striatum; Hip, hippocampus; Amy, amygdala. (**B**) X-Gal–stained coronal section of the hippocampus from double transgenic lines B lac1 and B lac2. CA1, CA1 cell body layer; CA3, CA3 cell body layer; DG, dentate gyrus.

Using in situ hybridization, we next examined the pattern of expression in three lines of double transgenic mice expressing tet-O–linked CaMKII-Asp^{286} (mouse lines B13, B21, and B22) (Fig. 3). In the first line (B13), expression was evident throughout the forebrain. However, in the hippocampus, expression was strong in the dentate gyrus and CA1 region but was weak or absent in the CA3 region. In a second line of mice (B22), there was moderate expression in the hippocampus, subiculum, striatum, and amygdala, with little expression in neocortex. In the hippocampus, expression was again present in the CA1 region and absent in the CA3 region. In the third line (B21), there was little expression in the neocortex and hippocampus but strong expression in the striatum, in anterior and lateral amygdala nuclei, and in the underlying olfactory tubercle. Thus, whereas the CaMKII promoter can limit expression to forebrain neurons generally, expression of the tet-O–linked transgene is further limited to particular subsets of forebrain neurons, presumably due to integration site–dependent effects.

In double transgenic mice, a high level of expression of the CaMKII-Asp^{286} mRNA was obtained (Figs. 1B and 3). To determine the effect of this expression on enzyme activity, we measured CaMKII activity in the striatum of the B21 line of mice (Table 1). In these mice, Ca^{2+}-independent CaMKII activity was increased sevenfold relative to that of the wild type. However, when the mice were treated with doxycycline (1 mg/ml), CaMKII activity was suppressed to wild-type values. When the doxycycline treatment was discontinued, Ca^{2+}-independent CaMKII activity returned to those of the untreated transgenic mice. Thus, the CaMKII-Asp^{286} transgene is functionally expressed and can be regulated with doxycycline.

Effects on LTP of CaMKII-Asp^{286} ex-

Table 1. Effect of CaMKII-Asp^{286} mRNA expression on enzyme activity. Brains were removed and the striatum was dissected and immediately homogenized in 20 mM tris-HCl (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA, 2 mM leupeptin, 0.4 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.4 mM molybdate, and 10 mM sodium pyrophosphate. CaMKII enzyme activity was determined as described (*7*). B21 + Dox animals received doxycycline (1 mg/ml) plus 5% sucrose in the drinking water for 3 to 5 weeks. B21 + Dox withdrawal animals received doxycycline (1 mg/ml) for 3 to 5 weeks and were then switched to normal water for 6 weeks. The number of mice is given in parentheses.

Mouse line	CaMKII activity		
	Without Ca^{2+} (pmol min^{-1} μg^{-1})	With Ca^{2+} (pmol min^{-1} μg^{-1})	Ca^{2+}-independent (%)
Wild type	0.13 ± 0.01 (5)	10.4 ± 1.2	1.33 ± 0.21
B21	0.90 ± 0.14 (5)	20.9 ± 2.9	4.62 ± 1.02
B21 + Dox	0.16 ± 0.04 (5)	12.9 ± 1.5	1.22 ± 0.30
B21 + Dox withdrawal	0.80 ± 0.03 (3)	14.2 ± 0.7	5.70 ± 0.43

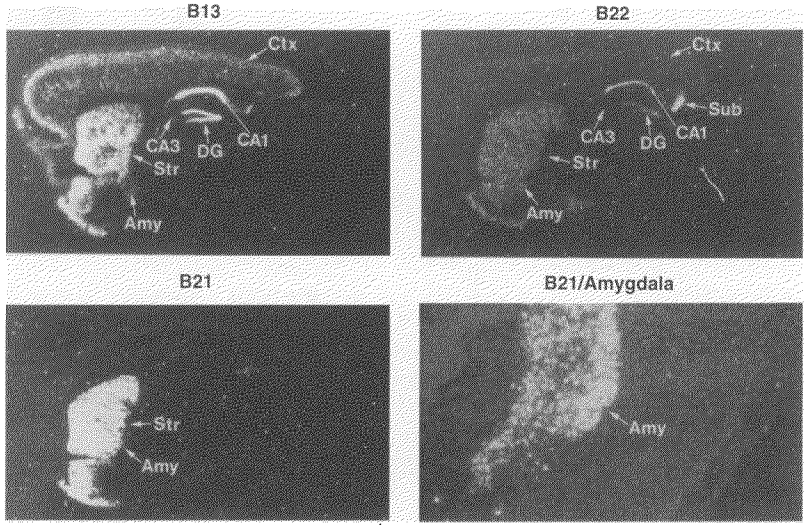

Fig. 3. Regional distribution of the CaMKII-Asp^{286} mRNA determined by in situ hybridization (*7*). Medial sagittal sections of double transgenic lines B13, B21, and B22 showing CaMKII-Asp^{286} transgene expression. B21/Amygdala shows a close-up view of a coronal section from the B21 double transgenic line of mouse.

pression in the hippocampus. Constitutive expression of the CaMKII-Asp^{286} transgene in the mouse forebrain shifts the stimulation frequency required for the production of LTP and LTD in the Schaffer collateral pathway of the hippocampus (*7*). In wild-type mice, stimulation at 1 Hz produced LTD, whereas stimulation at 5, 10, or 100 Hz produced LTP. In transgenic mice, stimulation at 100 Hz still produced LTP. However, stimulation in the 5- to 10-Hz range no longer produced LTP, but rather produced LTD or no change in synaptic strength.

We investigated whether the transgene was acting presynaptically or postsynaptically by asking whether expression of the transgene specifically in the postsynaptic CA1 neurons would produce a shift in the frequency threshold for LTP and LTD. We examined the B13 line of mice, which showed a uniformly high level of expression in the CA1 region, with little or no expression in CA3 (*22*). Thus, when Schaffer collateral LTP is measured in the B13 mice, the transgene will be expressed only in the postsynaptic neurons. Stimulation of slices from wild-type mice at 10 Hz resulted in a long-lasting potentiation of 123 ± 9% (n = 12 slices, 6 mice) (Fig. 4). By contrast, 10-Hz stimulation in B13 transgenic mice produced a slight depression to 89 ± 6% of baseline (n = 9 slices, 3 mice), which was significantly different from wild-type mice [$t(19) = 3.148$; $P < 0.01$, Student's t test].

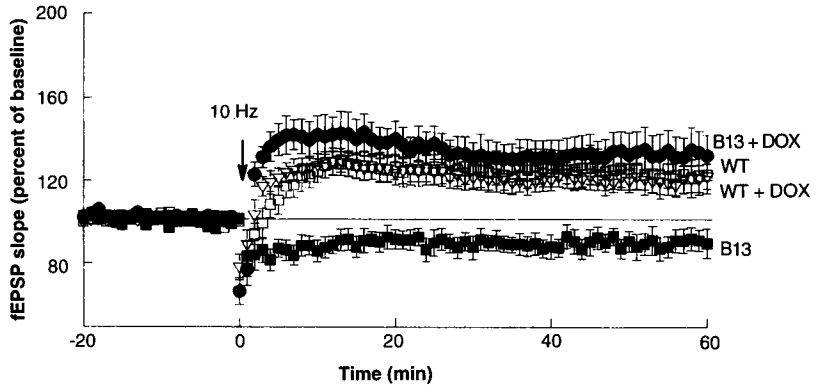

Fig. 4. Reversal of 10-Hz LTP deficit in CA1 of hippocampal slices. Field EPSP slopes before and after 10-Hz tetanic stimulation were recorded and expressed as the percentage of pre-tetanus baseline (*22*). Stimulation at 10 Hz for 1.5 min induced a transient depression followed by potentiation in wild-type mice (123 ± 9% at 60 min after tetanus; n = 12 slices, 6 mice) (□). Tetanus (10 Hz) induced a slight depression in B13 double transgenic mice (89 ± 6% at 60 min after tetanus; n = 9 slices, 3 mice) (■). Doxycycline treatment reversed the defect in B13 mice (132 ± 10%; n = 8 slices, 4 mice) (●). Doxycycline treatment had no effect on synaptic potentiation in wild-type mice (122 ± 6%; n = 16 slices, 6 mice) (▽).

To determine whether this effect was reversible, we suppressed transgene expression by administering doxycycline (1 mg/ml) for 2 to 3 weeks. Ten-hertz stimulation then produced potentiation similar to that in wild-type mice (132 ± 10%; n = 8 slices, 4 mice) (Fig. 4). Thus, suppression of transgene expression in adult mice reversed the electrophysiological phenotype [$t(15) = 3.675$, $P < 0.005$]. These results suggest that the selective expression of the CaMKII-Asp^{286} transgene in the postsynaptic CA1 neurons of the Schaffer collateral synapse is sufficient to alter the frequency threshold for LTP. Moreover, the shift in the frequency threshold is due to the acute expression of the transgene rather than to an irreversible developmental defect (*23*).

Effect on explicit memory storage of CaMKII-Asp^{286} expression in the hippocampus. Expression of the CaMKII-Asp^{286} transgene in the forebrain interferes with spatial memory, a form of explicit memory, as measured in the Barnes circular maze (*8*). The Barnes circular maze is a brightly lit open disk with 40 holes in the perimeter (Fig. 5A). Mice have an aversion for brightly lit open areas and hence are motivated to escape from the maze. This can be achieved by finding the 1 hole in 40 that leads to a darkened escape tunnel. In the spatial version of this task, the mouse must use distal cues in the room to locate the hole that leads to the escape tunnel (*24*).

Expression of the CaMKII-Asp^{286} transgene throughout the forebrain as seen in the B13 mice results in an impairment in the spatial but not the cued version of the Barnes maze task (*8*). To investigate those areas in the forebrain that are critical for this type of defect in spatial memory, we examined the B22 transgenic mice that show expression in the hippocampus, subiculum, striatum, and amygdala, but relatively little expression in the neocortex (Fig. 3). These mice exhibited significant impairment in spatial memory on the Barnes circular maze. None of the transgenic mice was able to acquire the task by using the spatial strategy, despite the fact that they were trained for 40 consecutive days (Fig. 5, B to D). Nevertheless, this profound memory impairment was reversed by suppression of transgene expression.

Effect on implicit memory of CaMKII-Asp^{286} expression in the amygdala and striatum. Fear conditioning is a simple associative form of learning, in which both a novel environment and a tone are paired

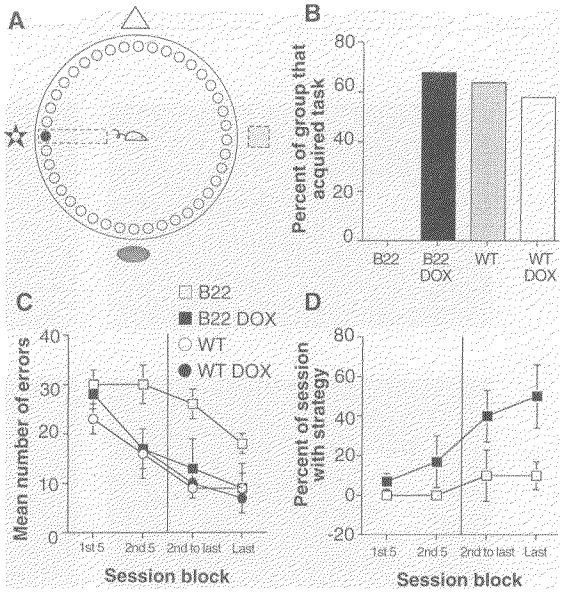

Fig. 5. Reversible deficits in explicit learning and memory in mice expressing the CaMKIIα transgene. (**A**) The Barnes circular maze. (**B**) Percentage of B22 transgenic and wild-type mice that met the learning criterion on the Barnes circular maze (*24*). A chi-square analysis revealed that the percentage of B22 transgenics acquiring the Barnes maze (0%) was significantly different from B22 transgenics on doxycycline and both wild-type groups ($X^2 = 53.05$, $P < 0.0001$). Four groups of mice were tested: B22 transgenics (n = 6), B22 transgenics on doxycycline (1 mg/ml) for 4 weeks (n = 6), wild types (n = 8), and wild types on doxycycline (1 mg/ml) for 4 weeks (n = 7). (**C**) Mean number of errors across session blocks composed of five sessions. Values represent group means ± SEM. A three-way ANOVA revealed a main effect of genotype [$F(1,23) = 4.28$, $P = 0.04$). (**D**) The percentage of sessions in which the spatial search strategy was used across session blocks by B22 transgenic mice. Values represent group means ± SEM. A two-way ANOVA revealed a significant main effect of doxycycline [$F(1,10) = 7.313$, $P = 0.02$).

with a foot shock on the training day (25). Memory is assessed 24 hours later by measurement of the amount of freezing (the fear response) elicited by either the novel environment (context conditioning) or the tone (cued conditioning). Fear conditioning shows components of both implicit and explicit forms of learning. The contextual version of the task is selectively impaired by lesions of the hippocampus (26) and thus can be viewed as an explicit form of learning, whereas both the cued and contextual versions of the task are impaired by lesion of the amygdala and are therefore viewed as implicit. In contrast to their spatial memory deficit, the B22 line of mice showed normal fear conditioning to both the cue and the context (Fig. 6, A and B). Thus, even though the B22 mice are impaired in spatial memory on the Barnes maze, they are not impaired in a second hippocampal-dependent task (contextual fear conditioning). This dissociation has been observed previously with constitutive expression of the CaMKII-Asp^{286} transgene and may reflect the use of different synaptic mechanisms for the storage of memory in the two tasks (7). In addition, these results demonstrate that the moderate level of transgene expression in the amygdala and striatum seen in the B22 mice (Fig. 3) is insufficient to interfere with the implicit component of fear conditioning.

Does a higher level of expression of the CaMKII-Asp^{286} transgene in the striatum and amygdala affect implicit memory storage? To explore this question, we studied the B21 mice that showed strong expression in the lateral amygdala and striatum but little transgene expression in the hippocampus or neocortex (Fig. 3). The B21 transgenic mice exhibited a severe impairment in both context and cued conditioning (Fig. 6, A and B). This learning impairment was again reversed by administration of doxycycline for 4 weeks before training.

This deficit in fear conditioning most likely arises from expression in the lateral amygdala, a structure that has been implicated in this form of learning by lesion studies (27). However, because there are many reciprocal connections between the striatum and the amygdala (28), we cannot rule out the possibility that the deficit results from a functional disruption in the striatum that secondarily alters the amygdala.

Effect on memory retrieval of CaMKII-Asp^{286} expression in the amygdala and striatum. Withdrawal of doxycycline after an initial period of transgene suppression resulted in a reactivation of gene expression (Table 1). We examined whether reexpression of the transgene, after normal learning has occurred, interferes with later stages of memory storage such as consolidation or retrieval. We trained B21 mice with the transgene expression suppressed and observed robust fear conditioning. Once the animals had learned the task, we reactivated transgene expression by withdrawing doxycycline (Fig. 6C). After a 6-week period, the expression of the CaMKII-Asp^{286} transgene returned to the same levels found in animals that had not received the drug (Table 1). We then examined these mice for retention of both context and cued conditioning and found a significant reduction in freezing compared to B21 mice in which we maintained suppression of the transgene (Fig. 6D).

This reduction in freezing reflects either an impairment in memory consolidation or recall, or a deficit in performance. The evaluation of performance deficits is critical to the study of memory because one can only infer that memory storage is defective once all possible defects in perception, motor performance, and cognitive understanding of the task have been excluded. Although it is difficult to control for all consequences of a genetic manipulation on various components of performance, we have examined the two most likely classes of performance variables: (i) the ability to perceive the unconditioned stimulus, and (ii) the ability to attend to and freeze in response to fearful stimuli (the conditioned response). To rule out an impairment in perception of the unconditioned stimulus (foot shock), we examined sensitivity to shock and found no difference between B21 transgenic and wild-type mice, suggesting that the observed fear-conditioning deficit did not result from a difference in the perception of the unconditioned stimulus (29). We next examined the possibility of a defect in performance of the conditioned response (freezing) by measuring unconditioned freezing in response to an intruder (30). We again found no difference in the ability of B21 mice to freeze to an intruder (a rat) when the transgene was expressed (Fig. 6E). Thus, the B21 transgenic mice were able to attend to fearful stimuli and to express a normal freezing response. Although some occult defect in performance might have been present that we have not detected, these control experiments argue that the transgene does not produce its effect on the perception of the unconditioned stimulus or on performance of the conditioned response. Rather, the results suggest that the CaMKII signaling pathway is important for some later aspects of memory storage such as the ability to consolidate or to recall the learned information.

Discussion. High levels of Ca^{2+}-inde-

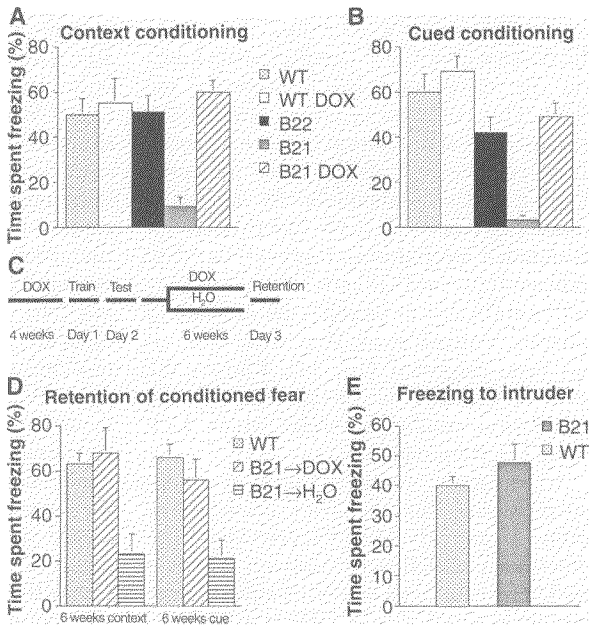

Fig. 6. Reversible deficits in implicit learning and memory in mice expressing the CaMKIIα transgene. Percentage of time spent freezing to context (**A**) and to cue (**B**) 24 hours after training in the B22 and B21 lines. Values represent group means ± SEM. A three-way ANOVA revealed a significant three-way interaction for context (genotype by line by doxycycline) ($F[1,55] = 9.177$, $P = 0.0037$) and a significant two-way interaction for cue (line by genotype) ($F[1,55] = 5.087$, $P = 0.0281$). Six groups of mice were tested: B22 transgenics ($n = 6$), B22 transgenics on doxycycline for 4 weeks ($n = 11$), B21 transgenics ($n = 8$), B21 transgenics on doxycycline for 4 weeks ($n = 19$), wild types (from both B22 and B21 lines) ($n = 11$), and wild types (from both B22 and B21 lines) on doxycycline for 4 weeks ($n = 8$). (**C**) Time line illustrating administration of doxycycline and behavioral training and testing. (**D**) Retention of context and cued conditioning. Percentage of time spent freezing to context and cue 6 weeks after training. Values represent group means ± SEM. Post hoc analysis by the Scheffe test revealed that B21 transgenic mice that were switched to water froze significantly less to context than B21 transgenic mice on doxycycline ($P = 0.01$) and wild types ($P = 0.008$) and significantly less to cue than B21 transgenic mice on doxycycline ($P = 0.02$) and wild types ($P = 0.0088$). Three groups of mice were tested: B21 transgenics on doxycycline for 4 weeks before training and 6 weeks after training ($n = 8$), B21 transgenics on doxycycline for 4 weeks before training that were switched to water for the 6 weeks after training ($n = 8$), and wild-type mice (from both B22 and B21 lines, $n = 19$). (**E**) The percentage of time spent freezing to an intruder during the first 120 s after the mouse was exposed to a rat. Values represent group means ± SEM.

pendent CaMKII activity shifted the stimulation-frequency threshold for hippocampal LTP and LTD to favor LTD (*7*). This shift in threshold is associated with an impairment in explicit, but not implicit, memory (*8*). To obtain regulated expression of this transgene in restricted regions of the forebrain so that we could study the underlying cellular and behavioral functions more effectively, we used the tTA system for regulated gene expression (*13*, *14*).

We found that expression of the CaMKII-Asp^{286} transgene altered adult synaptic plasticity and memory formation directly, and not by effects on neuronal development. In addition, expression of the transgene postsynaptically was sufficient to alter the frequency threshold for LTP induction, at least at 10 Hz. Finally, high-level activation of CaMKII in the striatum and lateral amygdala also interfered with implicit forms of memory.

How might an increase in Ca^{2+}-independent CaMKII activity alter the stimulation frequency required to produce LTP and LTD, and how might this in turn alter learning and memory storage? Our results demonstrate that the effect of the CaMKII-Asp^{286} transgene is likely mediated by changes in the postsynaptic CA1 neurons of the Schaffer collateral pathway. A simple mechanism for systematically shifting the frequency threshold for LTP and LTD to favor LTD would be to reduce the size of the postsynaptic Ca^{2+} signal produced during the stimulation [(*31*); however, see (*32*)]. This could occur either through the increased phosphorylation of particular substrate proteins of CaMKII or by increased binding of Ca^{2+}-calmodulin by autophosphorylated CaMKII (*33*). Independent of its detailed mechanisms, however, our data indicate that CaMKII activation alone may not be sufficient to produce the increase in synaptic strength associated with LTP, as has been suggested (*18*, *34*). Rather, the level of CaMKII activation regulates the stimulation conditions under which LTP and LTD are produced.

In this study, we did not measure synaptic physiology and behavior in the same group of animals (*35*). Nevertheless, the effects of CaMKII activation on behavior are likely a consequence of its effect on the frequency threshold for LTP and LTD induction. That CaMKII activation interferes with synaptic plasticity in the 5- to 10-Hz range is particularly relevant for the explicit hippocampal-based spatial memory paradigm. Animals exploring the space of a novel environment show a rhythmic oscillation in hippocampal activity in the 5- to 10-Hz range (the theta rhythm) (*19*). Changes in synaptic strength can be produced by this endogenous activity and are thought to be necessary for storing information about space. Synaptic plasticity in the theta frequency range may regulate hippocampal place cells, the pyramidal neurons (in the CA3 and CA1 subfields) whose activity is correlated with the animals' location in the environment (*36*).

Several lines of evidence implicate the lateral amygdala as the site of plasticity for fear conditioning. First, the lateral amygdala is the first site of convergence of somatosensory (unconditioned stimulus) and auditory (conditioned stimulus) information in the fear-conditioning pathway (*26*). Second, fear conditioning enhances the auditory-evoked responses of neurons in the lateral amygdala (*37*). Third, these neurons exhibit robust LTP that can contribute to enhanced auditory-evoked responses (*38*). Finally, lesions of the lateral amygdala block fear conditioning (*26*). How might the expression of CaMKII-Asp^{286} affect fear conditioning? Expression of this transgene in the hippocampus increases the stimulation frequency required to produce LTP (Fig. 4). Were a similar increase in the frequency threshold to occur at excitatory synapses in the lateral amygdala, this increase in threshold could form the physiological basis for the observed impairment in implicit memory storage.

Expression of the transgene in striatum and amygdala also affected memory consolidation or recall. Models of learning generally invoke changes in synaptic strength only during the initial learning process (*39*). Once formed, the changes in synaptic strength are thought to remain stable and to carry the actual memory trace. However, for some memories such as hippocampal-based explicit memories, the anatomical locus of the memory changes with time during a several-week period after the initial learning (*26*). Moreover, the recall of memory typically is reconstructive—it requires a new recapitulation of the learned experience. Both transfer and reconstruction of memory might require an activity-dependent change in synaptic strength. If a similar process occurs for fear conditioning in the amygdala, the defect in retrieval observed in the transgenic mice could reflect a defect in synaptic plasticity caused by CaMKII-Asp^{286} expression during this memory transfer or reconstruction phase.

The methods for regional and regulated transgene expression that we describe here represent initial steps toward the development of an optimal technology for the genetic study of cognitive processes. To carry the molecular dissection of behavior further, it will be necessary to use promoters that are even more restricted in their pattern of expression and to adapt this technology to the regulation of targeted gene disruption. With further modifications, the methods we describe here should prove generally useful and should help in elucidating the cellular and molecular signaling pathways important for higher cognitive processes.

REFERENCES AND NOTES

1. W. B. Scoville and B. Milner, *J. Neurol. Neurosurg. Psychiatry* **20**, 11 (1957); L. R. Squire, *Psychol. Rev.* **99**, 195 (1992).
2. D. L. Schacter and E. Tulving, *Memory Systems* (MIT Press, Cambridge, MA, 1994).
3. D. C. Blanchard and R. J. Blanchard, *J. Comp. Physiol. Psychol.* **81**, 281 (1972); M. Davis, in *The Amygdala: Neurobiological Aspects of Emotion, Memory, and Mental Dysfunction*, J. P. Aggleton, Ed. (Wiley-Liss, New York, 1992), p. 255; J. E. LeDoux, *ibid.*, p 339.
4. S. G. N. Grant *et al.*, *Science* **258**, 1903 (1992).
5. A. J. Silva, R. Paylor, J. M. Wehner, S. Tonegawa, *ibid.* **257**, 206 (1992).
6. A. J. Silva, C. F. Stevens, S. Tonegawa, Y. Wang, *ibid.*, p. 201.
7. M. Mayford, J. Wang, E. R. Kandel, T. J. O'Dell, *Cell* **81**, 891 (1995).
8. M. E. Bach, R. D. Hawkins, M. Osman, E. R. Kandel, M. Mayford, *ibid.*, p. 905.
9. M. Mayford, T. Abel, E. R. Kandel, *Curr. Opin. Neurobiol.* **5**, 141 (1995).
10. M. Gossen and H. Bujard, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5547 (1992).
11. P. A. Furth *et al.*, *ibid.* **91**, 9302 (1994).
12. S. G. Miller and M. B. Kennedy, *Cell* **44**, 861 (1986).
13. K. E. Burgin *et al.*, *J. Neurosci.* **10**, 1788 (1990).
14. P. I. Hanson and H. Schulman, *Annu. Rev. Biochem.* **61**, 559 (1992).
15. Y.-L. Fong, W. L. Taylor, A. R. Means, T. R. Soderling, *J. Biol. Chem.* **264**, 16759 (1989).
16. G. Thiel, A. J. Czernik, F. Gorelick, A. C. Nairn, P. Greengard, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6337 (1988).
17. R. Waldmann, P. I. Hanson, H. Schulman, *Biochemistry* **29**, 1679 (1990).
18. J. Lisman, *Trends Neurosci.* **17**, 406 (1994).
19. B. H. Bland, *Prog. Neurobiol.* **26**, 1 (1986).
20. The CaMKIIα promoter consisted of 8.5 kb of genomic DNA upstream of the transcription initiation site of the mouse CaMKIIα gene, as well as 84 base pairs of the 5′ noncoding exon. Genomic DNA was isolated from a C57 B16/J mouse spleen cosmid library with a rat genomic probe consisting of a 0.4-kb Ava I fragment comprising the transcription-initiation region of rat CaMKIIα (*40*). The tTA gene from plasmid pUHD 15-1 (*10*) was flanked by an artificial intron and splice sites at the 5′ end (*41*) and by a polyadenylation signal from SV40 at the 3′ end. The cDNA with intron and polyadenylation signal was placed downstream of the 8.5-kb CaMKII promoter fragment. The cDNAs for *Escherichia coli lacZ* and mouse CaMKIIα were similarly flanked by the hybrid intron and polyadenylation signal and placed downstream of the tet-O promoter element of plasmid pUHD 10-3 (*10*). The CaMKIIα gene was a full-length cDNA (4.8 kb) isolated from a C57B16/J mouse brain cDNA library. The *lacZ* gene carried an SV40 large T antigen nuclear localization signal as well as the 3′ untranslated region (UTR) of CaMKIIα, which targets the mRNA to dendrites (*42*).
21. RT-PCR was performed essentially as described (*7*). Total forebrain RNA (100 ng) was used in each reaction with oligonucleotide primers to amplify a region of the transcript that includes the Thr^{286}→Asp mutation. Equal amounts of amplified cDNA (both wild-type and mutant sequences) were separated on a 3% agarose gel, transferred to nylon membranes, and hybridized with a ^{32}P-labeled oligonucleotide probe specific for the Asp^{286} mutation (oligonucleotide sequence: 5′-CTTCAGGCAGTCGACGTC-CTCCTGTCTGTG-3′). Blots were washed under conditions in which only the Asp^{286} mutant cDNA

was detected (2′ 15 min, 60°C, 0.2′ standard saline citrate). A Northern (RNA) blot of total forebrain mRNA revealed expression of a shorter-than-expected CaMKII-Asp^{286} transcript (~3.4 kb). As shown in Fig. 3, this shorter CaMKII-Asp^{286} transcript did not localize to dendrites, presumably as a result of the loss of a sequence element in the 3′ UTR that is necessary for mRNA targeting to dendrites (*42*).

22. Transverse slices (400 μm thick) of mouse hippocampus were prepared and placed in an interface slice chamber perfused with artificial cerebrospinal fluid as described (*7*). Field excitatory postsynaptic potentials (EPSPs) were elicited once per minute with fine tungsten bipolar stimulation electrodes (0.05-ms pulse duration). Stainless steel recording electrodes were placed in striatum radiatum. The stimulation strength was set to produce 50% of the maximum obtainable EPSP in each slice. Baseline synaptic response was collected for 20 min before the tetanus. The 10-Hz tetanus was delivered for 1.5 min at the same intensity as used in the baseline recording. For doxycycline treatment, animals were administered doxycycline (1 mg/ml) plus 5% sucrose in the drinking water for 2 to 3 weeks, and the slices were the exposed to doxycycline (1 ng/ml) in the perfusate. All animals were 2.5 to 6 months of age at the time of recording.
23. It would also be useful to suppress transgene expression during development and then activate the gene only in the adult animal. However, we found that treatment of wild-type mice with doxycycline (1 mg/ml) during development impaired adult spatial memory and memory for fear conditioning. This result suggests that doxycycline itself produces a defect in neuronal development. We therefore used transgene suppression only in the adult animal in which the doxycycline treatment did not affect memory. Given the activation of the transgene throughout development, it is possible that the LTP and memory phenotypes observed with the transgene active in the adult animal result from a synergistic interaction between developmental and adult expression rather than a direct acute effect of transgene expression in the adult animal.
24. On the Barnes circular maze (*8*), the mice (2.5 to 6 months of age) were tested once a day until they met the criterion (five out of six sessions with three or fewer errors) or until 40 days had elapsed. The order of holes searched was recorded by an observer who was blind to genotype and doxycycline condition, and from these data the number of errors was determined. Errors were defined as searches of any hole that did not have the tunnel beneath it. Searches included nose pokes and head deflections over the hole. At the end of each session the search strategy used was recorded by the observer. The spatial search strategy was operationally defined as reaching the escape tunnel with both error and distance scores ≤3. Distance was calculated by counting the number of holes between the first hole searched within a session and the escape tunnel. A one-factor analysis of variance (ANOVA) (gender) revealed no significant effect of gender for either transgenic or wild-type mice, so the data were collapsed across this variable. For the error data, a three-factor ANOVA (genotype, doxycycline, and session block) with one repeated measure was used. For the spatial search strategy data, the two groups of B22 transgenic mice were compared with a two-way ANOVA (doxycycline and session block) with one repeated measure.
25. In the conditioned fear task (*8*), freezing was defined as a total lack of movement with the exception of respiration and was measured by an experimenter who was blind to genotype and doxycycline condition. The percentage of time spent freezing to context and cue was calculated. No significant effect of gender was observed in the B22 or B21 transgenic mice or the wild-type mice, so the data were collapsed across this variable. Freezing to context and cue on testing day was analyzed by two three-factor ANOVAs (genotype, line, and doxycycline) that were used to compare the B22 and B21 transgenic and wild-type mice. Two one-way ANOVAs were used to compare the amount of freezing 6 weeks later to cue and context in B21 transgenics on doxycycline, B21 transgenics switched to water, and wild-type mice.
26. J. J. Kim and M. S. Fanselow, *Science* **256**, 675 (1992).
27. J. E. LeDoux, P. Cicchetti, A. Xagoravis, L. M. Romanski, *J. Neurosci.* **10,** 1062 (1990).
28. H. Kita and S. T. Kitai, *J. Comp. Neurol.* **298**, 40 (1990); N. S. Canteras, R. B. Somerly, L. W. Swanson, *ibid.* **360**, 213 (1995).
29. Pain sensitivity was measured in B21 transgenic (n = 4) and wild-type mice (n = 6). The mice were placed individually in a mouse operant chamber with a metal grid floor and given 1-s foot shocks of increasing intensity (for example, 1, 2, 3, 4 mA . . .). An experimenter who was blind to the genotype of the mice recorded the intensity of foot shock required to elicit each of the following three responses: startles, vocalizations, and jumps. A t test for each response revealed no significant effect of genotype.
30. Unconditioned freezing in the presence of an intruder was measured in B21 transgenic (n = 8) and wild-type mice (n = 10) in a Nalgene plastic metabolism cage. The mice and intruder were placed in the upper and lower chambers, respectively. The chambers were separated by a metal grid floor. A seven-week-old male Sprague-Dawley rat served as the intruder and was placed in the lower chamber 10 min before introduction of the mouse. The amount of unconditioned freezing occurring during the first 120 s after the mouse was introduced was measured by an experimenter who was blind to genotype. A t test revealed no significant effect of genotype.
31. J. A. Cummings, R. M. Mulkey, R. A. Nicoll, R. C. Malenka, *Neuron* **16**, 825 (1996).
32. D. Neveu and R. S. Zucker, *ibid.*, p. 619.
33. T. Meyer, P. I. Hanson, L. Stryer, H. Schulman, *Science* **256**, 1199 (1992).
34. D. L. Pettit, S. Perlman, R. Malinow, *ibid.* **266**, 1881 (1994); P.-M. Lledo *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 11175 (1995).
35. The expression of the CaMKII-Asp^{286} transgene in the CA1 region of the B22 mice was patchy; that is, some neurons expressed the transgene well, whereas in other neurons expression was absent. This patchy expression precluded an assessment of LTP in this line of mice by means of field recordings, which sample many synapses from different neurons in a region. However, it is assumed that in those neurons where the transgene was strongly expressed in these mice, a shift in the LTP/LTD frequency threshold would occur.
36. J. O'Keefe and L. Nadel, *The Hippocampus as a Cognitive Map* (Oxford Univ. Press, New York, 1978).
37. G. J. Quirk, C. Repa, J. E. LeDoux, *Neuron* **15,** 1029 (1995).
38. M. T. Rogan and J. E. LeDoux, *ibid.*, p. 127.
39. P. S. Churchland and T. J. Sejnowski, *The Computational Brain* (MIT Press, Cambridge, MA, 1992).
40. T. Sunyer and N. Sahyoun, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 278 (1990).
41. T. Choi, M. Huang, C. Gorman, R. Jaenisch, *Mol. Cell. Biol.* **11**, 3070 (1991).
42. M. Mayford, D. Baranes, K. Podsypanina, E. R. Kandel, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 13250 (1996).

43. We thank R. Axel and T. Jessell for critically reading this manuscript; J. Finkelstein for maintaining and genotyping the mice; R. Shih, V. Winder, and L. Varshavsky for help with behavioral experiments; C. Lam for help with figures; H. Ayers and I. Trumpet for typing the manuscript; and M. Osman for animal care. This research was supported by the Howard Hughes Medical Institute and the National Institute of Mental Health.

8 August 1996; accepted 23 October 1996

EXHIBIT 2

Cell, Vol. 81, 891–904, June 16, 1995, Copyright © 1995 by Cell Press

CaMKII Regulates the Frequency-Response Function of Hippocampal Synapses for the Production of Both LTD and LTP

Mark Mayford, Jian Wang, Eric R. Kandel, and Thomas J. O'Dell*
Howard Hughes Medical Institute
Center for Neurobiology and Behavior
College of Physicians and Surgeons of Columbia University
New York, New York 10032

Summary

To investigate the function of the autophosphorylated form of CaMKII in synaptic plasticity, we generated transgenic mice that express a kinase that is Ca^{2+} independent as a result of a point mutation of Thr-286 to aspartate, which mimics autophosphorylation. Mice expressing the mutant form of the kinase show an increased level of Ca^{2+}-independent CaMKII activity similar to that seen following LTP. The mice nevertheless exhibit normal LTP in response to stimulation at 100 Hz. However, at lower frequencies, in the range of 1–10 Hz, there is a systematic shift in the size and direction of the resulting synaptic change in the transgenic animals that favors LTD. The regulation of this frequency-response function by Ca^{2+}-independent CaMKII activity seems to account for two previously unexplained synaptic phenomena, the relative loss of LTD in adult animals compared with juveniles and the enhanced capability for depression of facilitated synapses.

Introduction

Long-lasting increases and decreases in synaptic strength in response to different patterns of synaptic activation are thought to form the basis for the neuronal changes that contribute to various forms of learning and memory. In the hippocampus, a structure that is important for the storage of explicit forms of memory, two types of long-term synaptic plasticity have been demonstrated: long-term potentiation (LTP) and long-term depression (LTD) (see Bliss and Collingridge, 1993; Bear and Malenka, 1994, for review). In the CA1 region of the hippocampus, both LTP and LTD can be induced at the same synapses by different frequencies of stimulation; low frequencies induce LTD, whereas high frequencies induce LTP (Dudek and Bear, 1992, 1993). Although the precise molecular mechanisms underlying these two forms of plasticity are unclear, both require influx of Ca^{2+} into the postsynaptic cell through N-methyl-D-aspartate (NMDA) receptors (Lynch et al., 1983; Malenka et al., 1992; Mulkey and Malenka, 1992; Artola and Singer, 1993). The different levels of postsynaptic Ca^{2+} influx achieved by different frequencies of presynaptic activity are thought to activate opposing changes in protein phosphorylation, with the higher frequencies activating protein kinases necessary for LTP (Malenka et al., 1989; Malinow et al., 1989) and the lower frequencies activating protein phosphatases necessary for LTD (Mulkey et al., 1993, 1994; O'Dell and Kandel, 1994).

CaMKII is a multisubunit Ca^{2+}/calmodulin-dependent serine–threonine protein kinase whose activity is required for LTP (see Hanson and Schulman, 1992, for review). Injection of specific peptide inhibitors of CaMKII into the postsynaptic neuron blocks the induction of LTP (Malenka et al., 1989; Malinow et al., 1989). Moreover, mice lacking the α subunit of CaMKII also lack LTP and have a deficit in spatial learning (Silva et al., 1992a, 1992b). Exactly how CaMKII contributes to LTP is not known. Purified CaMKII is inactive in the absence of Ca^{2+}. However, upon exposure to Ca^{2+}/calmodulin, CaMKII is activated and can phosphorylate numerous target proteins, including itself, at several sites (Miller and Kennedy, 1986). The autophosphorylation of CaMKII at one site, Thr-286, is particularly important because it is both necessary and sufficient to produce an autoactive kinase, a kinase that is active even in the absence of Ca^{2+} (Schworer et al., 1988; Miller et al., 1988; Thiel et al., 1988; Lou and Schulman, 1989). The ability to convert to a Ca^{2+}-independent state following a brief exposure to Ca^{2+} has led to the suggestion that CaMKII may function as a memory molecule in LTP (Miller and Kennedy, 1986; Lisman, 1989, 1994). According to this view of LTP, the initial Ca^{2+} influx caused by high frequency stimulation activates CaMKII, which phosphorylates substrate proteins that initiate LTP. Once turned on, the kinase maintains its active state by continued autophosphorylation, even after Ca^{2+} has returned to its basal level. The CaMKII maintained in its active Ca^{2+}-independent state by autophosphorylation in turn continuously phosphorylates critical substrate proteins that then maintain LTP. In this case, the kinase functions as a simple on–off switch not only for the initiation but also for the maintenance of LTP. In support of this idea, LTP-inducing stimuli or strong depolarization of the postsynaptic cell leads to an increase in the level of Ca^{2+}-independent CaMKII activity in the hippocampal slice (Fukunaga et al., 1993; Bading et al., 1993; Ocorr and Schulman, 1991). Also, infection of hippocampal slices with a vaccinia virus carrying a truncated, Ca^{2+}-independent CaMKII gene potentiates synaptic transmission and blocks LTP induction (Pettit et al., 1994).

To test the role of CaMKII autophosphorylation in synaptic plasticity, we took advantage of a mutation in the kinase that mimics autophosphorylation at Thr-286. In this mutant form of CaMKII, Thr-286 is replaced with an acidic aspartate group (CaMKII–Asp-286). The mutant kinase is 20- to 30-fold more active in the absence of Ca^{2+} than the wild-type enzyme (Fong et al., 1989; Waldmann et al., 1990). Using the CaMKII α promoter to limit expression to the forebrain, we generated transgenic mice that express CaMKII–Asp-286 in the hippocampus at a level of Ca^{2+} independence greater than that reached during LTP. We then examined LTP and found that, at this level of Ca^{2+}-

*Present address: Department of Physiology, University of California, Los Angeles, School of Medicine, Los Angeles, California 90024.

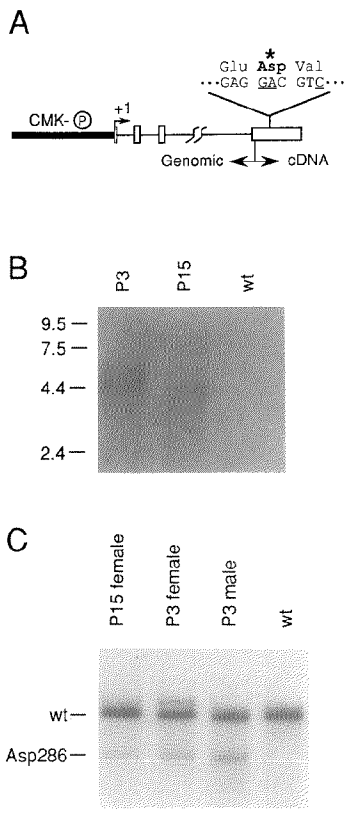

Figure 1. Expression of the CaMKII–Asp-286 Transgene

(A) Schematic representation of the CaMKII–Asp-286 construct used to generate transgenic mice.

(B) Northern blot of 20 μg of total hippocampal RNA from transgenic and wild-type animals probed with an Asp-286-specific oligonucleotide.

(C) RT–PCR quantitation of the relative level of transgene versus wild-type mRNA expression in total hippocampus.

independent activity, the kinase does not act as a simple on–off switch. Rather, the Ca^{2+}-independent kinase regulates the frequency–response function that couples presynaptic activity of the synapse to the production of either LTP or LTD. A high level of Ca^{2+}-independent activity favors the production of LTD even at frequencies that normally produce LTP.

Results

Generation of Mice Carrying a Transgene with a Thr-286→Asp Mutation in CaMKII

The α subunit of CaMKII is the most abundantly expressed isoform in forebrain structures such as hippocampus (McGuinness et al., 1985; Miller and Kennedy, 1985), and gene targeting experiments show that it is critical for the induction of LTP (Silva et al., 1992a). We therefore used the α subunit gene of CaMKII for the generation of transgenic mice. To limit the transgene to only specific target regions of the forebrain, we expressed the transgene under the control of its own promoter. We obtained 8.5 kb upstream of the CaMKIIα transcription initiation site

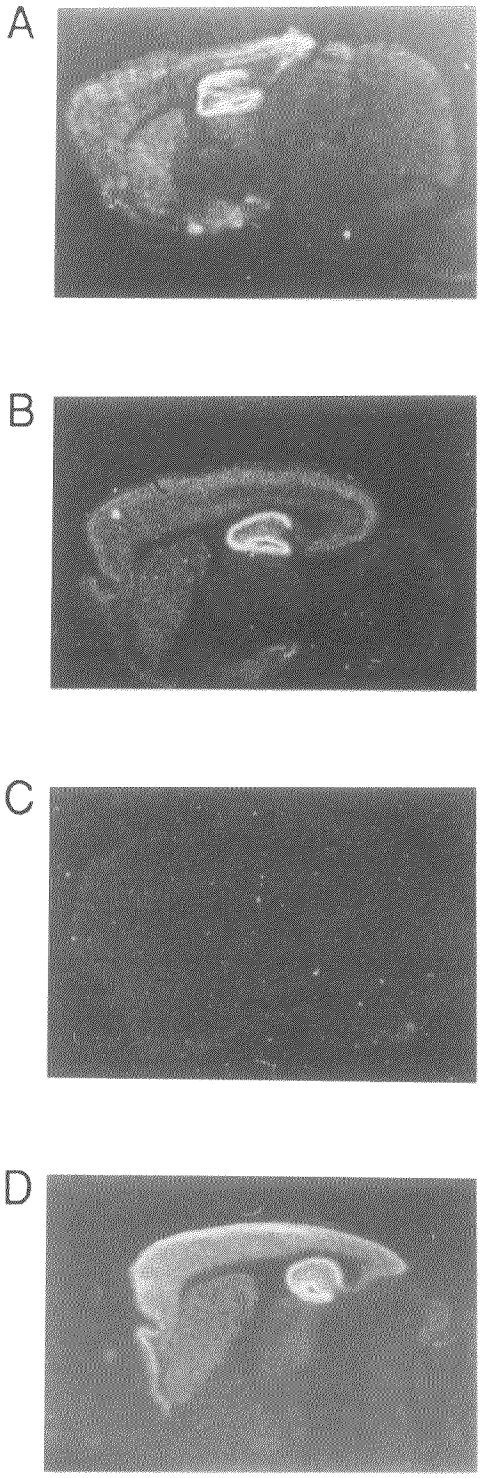

Figure 2. Regional Distribution of CaMKII–Asp-286 Transgene Expression by In Situ Hybridization

Sagittal sections from transgenic and wild-type animals hybridized with probes specific for the CaMKII–Asp-286 transgene or wild-type CaMKIIα. (A) P3 mouse, Asp-286 probe; (B) P15 mouse, Asp-286 probe; (C) wild-type mouse, Asp-286 probe; (D) wild-type mouse, wild-type probe.

Table 1. CaMKII Enzyme Assays

	Without CA^{2+} (pmol/min/μg)	With Ca^{2+} (pmol/min/μg)	Ca^{2+}-Independent (%)
Adult (+PO_4)			
P3	1.25 ± 0.29 (n = 6)[a]	8.75 ± 1.27	13.4 ± 1.5[c]
P15	1.00 ± 0.19 (n = 6)[a]	7.08 ± 0.94	14.6 ± 2.7[a]
Wild type	0.60 ± 0.09 (n = 10)	7.90 ± 0.72	7.4 ± 0.6
Young (+PO_4)			
P3	2.72 ± 0.55 (n = 4)[b]	10.23 ± 2.63	28.2 ± 2.6[c]
P15	1.28 ± 0.27 (n = 4)	6.82 ± 2.04	20.1 ± 1.9[a]
Wild type	1.05 ± 0.22 (n = 9)	8.70 ± 0.87	11.7 ± 1.7
Adult (−PO_4)			
P3	0.351 ± 0.039 (n = 6)[c]	6.74 ± 1.02	6.06 ± 1.41[b]
P15	0.406 ± 0.023 (n = 6)[c]	9.53 ± 0.67[b]	4.39 ± 0.41[c]
Wild type	0.076 ± 0.008 (n = 6)	5.77 ± 0.71	1.47 ± 0.30
Young (−PO_4)			
P3	0.654 ± 0.059 (n = 3)[c]	13.95 ± 1.23	4.73 ± 0.44[c]
P15	0.325 ± 0.048 (n = 4)[b]	10.10 ± 1.27	3.19 ± 0.15[c]
Wild type	0.058 ± 0.013 (n = 3)	8.95 ± 1.70	0.64 ± 0.03

Enzyme activity determined from total hippocampal extracts from adult (2- to 8-month-old) or young (3- to 4-week-old) mice. All values are mean ± SEM. For statistical comparisons, transgenic (P3 or P15) values were compared with wild-type values within the same group using a Student's t test. +PO_4, assayed in the presence of protein phosphatase inhibitors; −PO_4, assayed in the absence of protein phosphatase inhibitions.
[a] $p < .05$.
[b] $p < .01$.
[c] $p < .001$.

(Sunyer and Sahyoun, 1990) as well as a 5′ portion of the coding region from a mouse genomic library. We then isolated a mouse cDNA clone that contained the entire 3′ untranslated region, which we have found to be necessary for appropriate targeting of the CaMKIIα mRNA to dendrites (M. M. and E. R. K., unpublished data). Using oligonucleotide-directed mutagenesis, we introduced an aspartate into the cDNA at codon 286. The CaMKIIα cDNA containing the Thr-286→Asp mutation was then fused in phase to the corresponding exon of the genomic clone (Figure 1A). Transgenic mice were generated using this construct, and 7 independent founder animals were obtained, of which 3 reproduced successfully and transmitted the transgene. Two of these lines, referred to as P3 and P15, were analyzed in detail for the present study.

The Transgene Is Expressed in a Restricted Manner

By Southern blot analysis, we found a copy number of 1 for the P15 line and about 4 for the P3 line (data not shown). Breeding indicated that the transgene was X-linked in the P3 line. Northern blot analysis of hippocampal RNA, using an oligonucleotide probe specific for the Asp-286 mutant form of CaMKII, revealed the expression in the transgenic mice of a transcript of about 4.8 kb (Figure 1B). We used the reverse transcription–polymerase chain reaction (RT–PCR) to examine the relative level of CaMKII–Asp-268 mRNA as compared with the endogenous wild-type CaMKII mRNA (Figure 1C). In P15 hippocampus the transgene was expressed at 10% of wild-type levels, whereas in the P3 line expression was 16% (females) or 21% (males) of wild-type levels.

We examined the distribution of neuronal expression of the transgene by in situ hybridization using an oligonucleotide probe specific for the Asp-286 mutation. In both lines, the transgene was expressed selectively in forebrain regions, in a pattern that was indistinguishable from that of the endogenous CaMKIIα gene, except that the transgene appeared to be reduced or absent from a medial layer of the cortex that normally expresses endogenous CaMKIIα (Figure 2). The transgene was found throughout the dendritic layers of the hippocampus, suggesting that the mRNA was properly targeted to dendrites.

To determine whether the CaMKII–Asp-286 protein was functionally expressed, we compared CaMKII activity in hippocampal extracts from transgenic and nontransgenic litter mates. We measured the kinase activity in crude homogenates against autocamtide-2, a peptide substrate specific for CaMKII (Ocorr and Schulman, 1991). The transgenics showed a significant elevation in the Ca^{2+}-independent kinase activity without a corresponding elevation in the Ca^{2+}-dependent kinase activity, leading to an increase in the fraction of kinase in the Ca^{2+}-independent state (Table 1). The level of Ca^{2+}-independent activity measured in both transgenic lines was similar and nearly double that of wild-type animals (13.4% ± 1.5% [P3] and 14.6% ± 2.7% [P15] versus 7.4% ± 0.6% [wild type]). This level of kinase activation is greater than that observed following LTP induced by tetanic stimulation (Fukunaga et al., 1993) and equal to that produced by global depolarizing stimuli (Ocorr and Schulman, 1991; Bading et al., 1993).

The basal Ca^{2+}-independent CaMKII activity in the hippocampus is normally maintained by autophosphorylation of the endogenous enzyme. To determine whether the elevated kinase activity in the transgenic mice was due to expression of the mutant form of the enzyme and to provide an estimate of the total amount of transgene expression, we measured the activity in the absence of protein phosphatase inhibitors (Table 1). Under these conditions, phosphorylation of the endogenous CaMKII is

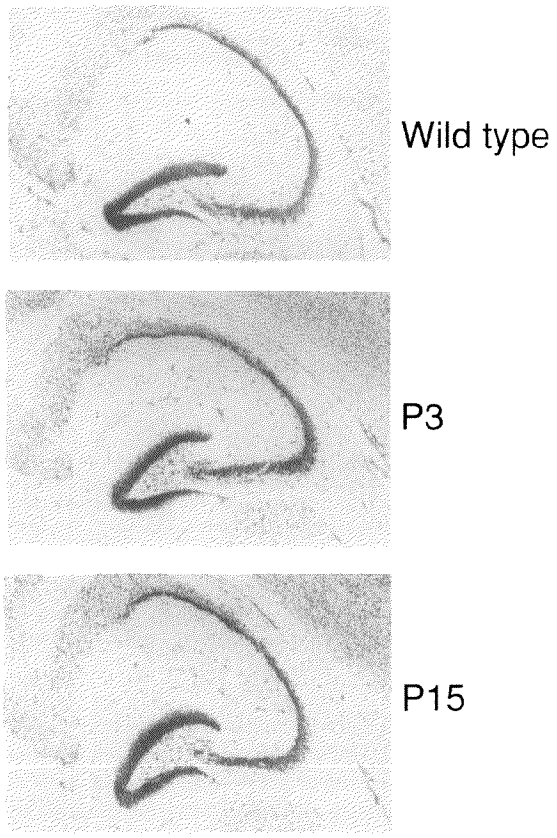

Figure 3. Nissl-Stained Sections from Wild-Type and CaMKII–Asp-286 Mice

Horizontal sections (20 μm) were stained as described by Grant et al. (1992).

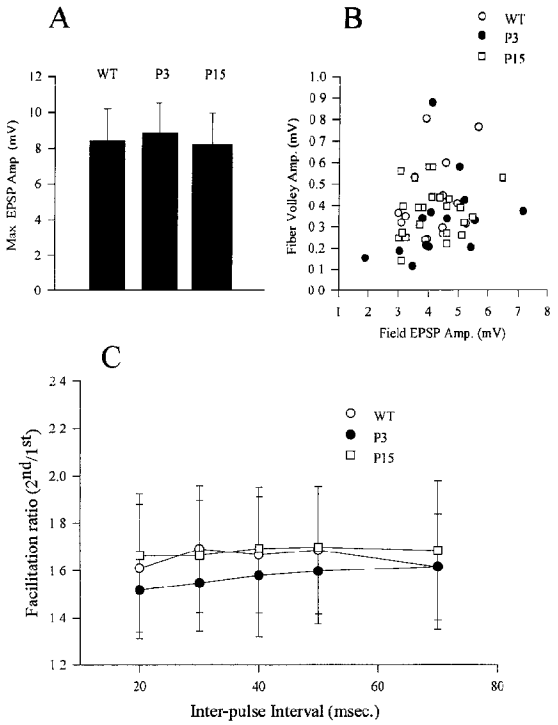

Figure 4. Basic Synaptic Physiology

(A) The maximal EPSPs determinable from slices from wild-type (WT) and transgenic (P3 and P15) animals were not significantly different. Values are as follows (mean ± SD): 8.39 ± 1.79 mV for wild type (n = 43 animals, 164 slices), 8.79 ± 1.69 mV for P3 (n = 32 animals, 115 slices), 8.18 ± 1.75 mV for P15 (n = 30 animals, 93 slices). Wild type versus P3: t(73) = 1.09, not significant; wild type versus P15: t(71) = 0.40, not significant.

(B) The EPSP versus fiber volley in millivolts measured at a stimulating intensity that produces 50% of the maximal EPSP. No significant difference was observed in the EPSP size or fiber volley between wild types and transgenics. Fiber volleys were 0.427 ± 0.19 mV for wild type (n = 7 animals, 14 slices), 0.337 ± 0.20 mV for P3 (n = 14 animals, 67 slices), and 0.378 ± 12 mV for P15 (n = 12 animals, 23 slices). Wild type versus P3: t(26) = 1.24, not significant; wild type versus P15: t(35) = 0.78, not significant. EPSPs were 4.13 ± 0.8 mV (wild type), 4.38 ± 1.27 mV (P3), and 4.16 ± 0.91 mV (P15). Wild type versus P3: t(26) = 0.62, not significant; wild type versus P15: t(35) = 0.12, not significant.

(C) Paired stimulation pulses were delivered at five interpulse intervals, and the ratio of the EPSP amplitude of the second to the first pulse was measured. No significant differences between wild-type and transgenic mice were observed at any of the interpulse intervals measured.

eliminated, and the level of Ca^{2+}-independent activity is dramatically reduced. The remaining difference between wild-type and transgenic animals reflects the presence of the CaMKII–Asp-286 protein, whose activity is not affected by phosphorylation state. These data allow us to estimate the relative amount of mutant enzyme in the hippocampal extracts. Assuming that the CaMKII–Asp-286 enzyme has 35% Ca^{2+}-independent activity (Waldmann et al., 1990), the percentage of total enzyme in the hippocampal extract that derives from the mutant protein is 9% for the P15 line and 14% for the females of the P3 line. This is consistent with the estimate of mRNA expression in the two lines. Since the level of expression of the transgene is only 10%–15% of the wild-type gene, we expect that the phenotypic effects we observe result from the Ca^{2+}-independent nature of the enzyme rather than from simple overexpression.

Gross Neuroanatomy and Behavior

In the P3 line, about 50% of the males died by 8 weeks of age and in many cases were observed to have what appeared to be severe seizures. Based on its transmission pattern, the transgene in this line seemed to be X-linked, suggesting that the seizures could be the result of insertional inactivation of a critical gene on the X chromosome. The males from the P3 line were therefore not analyzed further in the present study. The P3 females showed normal viability rates, and only 1 out of about 100 animals was observed to have seizures. The P15 line displayed normal viability, and we never observed seizures in this line. The P3 females as well as P15 males and females were indistinguishable from wild-type animals when observed during routine handling. However, the females from both lines failed, in most cases, to rear their young.

Since expression of the transgene might alter neuronal development, we examined Nissl-stained horizontal sec-

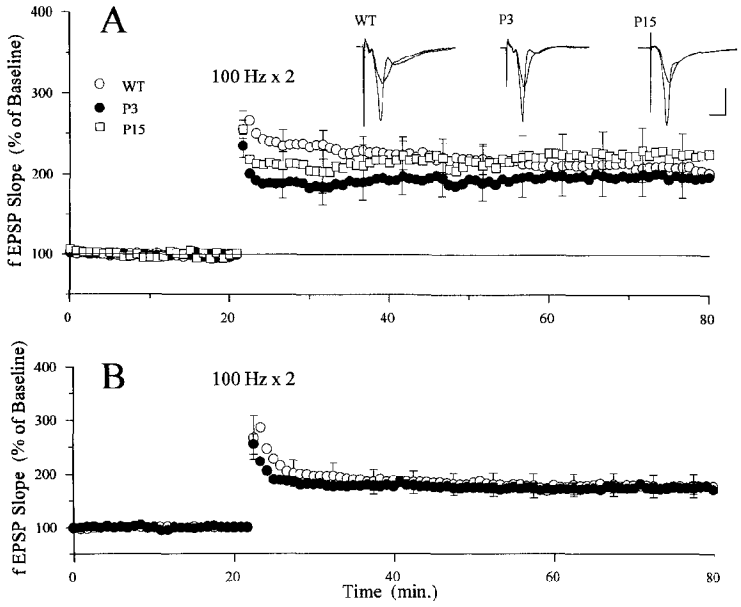

Figure 5. Induction of CA1 LTP by High Frequency Stimulation

(A) LTP induced by two 100 Hz, 1 s trains produced by shocks delivered at an intensity that produces 50% of the maximal EPSP level. The two trains were delivered 10 s apart. Recording was also at 50% of the maximal EPSP. Values are the percentage of prestimulation baseline measured 1 hr after stimulation (mean ± SEM; n = number of animals): 205% ± 18% (wild type; n = 7); 189% ± 20% (P3; n = 8), and 203% ± 28% (P15; n = 6). Wild type versus P3: t(13) = 0.59, not significant; wild type versus P15: t(11) = 0.74, not significant. Traces show individual field EPSPs evoked just prior to (smaller response) and 60 min after (larger response) the induction of LTP. Bars, 4 mV, 4 ms.

(B) LTP produced by two 100 Hz, 1 s trains delivered at 75% of the maximal EPSP level. The two trains were delivered 20 s apart. Recording was at 25% of maximal EPSP (wild type: 173% ± 20% [n = 4]; P3: 172% ± 13% [n = 3]; t(5) = 0.031, not significant).

tions. We found no difference in the arrangement of cell bodies within the neuronal layers of the hippocampus between transgenics from either line and control littermates (Figure 3), suggesting that transgene expression does not produce any gross developmental abnormalities.

LTP Is Normal in CaMKII-Asp-286 Mice

We next investigated the effect of CaMKII–Asp-286 expression on synaptic transmission and synaptic plasticity in the hippocampus, by recording extracellular field potentials produced in the stratum radiatum of hippocampal slices from P3 and P15 animals following Schaffer collateral stimulation.

We first compared basal synaptic transmission in hippocampal slices of wild-type and CaMKII–Asp-286 transgenic animals and found no difference in either the size of the field excitatory postsynaptic potential (EPSP; Figure 4A) or the fiber volley required to produce this EPSP (Figure 4B) in the transgenic animals as compared with wild types. This suggests that synaptic transmission is not substantially altered by the introduction of the CaMKII–Asp-286 transgene. We also examined paired-pulse facilitation in the transgenic mice. Paired-pulse facilitation is a short-lasting (<1 s) enhancement in synaptic strength that is thought to be mediated by a presynaptic mechanism (Kamiya and Zucker, 1994). There were no significant differences in paired-pulse facilitation from transgenic and wild-type animals (Figure 4C). Thus, we did not detect any change in the presynaptic processes that are thought to be regulated by CaMKII (Llinás et al., 1985; Lin et al., 1990; Nichols et al., 1990; Silva et al., 1992a).

We next asked whether CaMKII serves as a simple on–off switch for the generation of LTP. If so, the elevated kinase present in the transgenic animals in the basal state, which is equal to or greater than that present in wild-type animals following strong depolarizing stimuli that induce LTP (Ocorr and Schulman, 1991; Fukunaga et al., 1993; Bading et al., 1993), should switch LTP on in the basal state and thereby occlude further attempts to elicit LTP. To test this idea, we used a standard 100 Hz tetanus protocol that produces saturating amounts of LTP in adult wild-type animals. We found no difference in either the amplitude or duration of LTP between wild-type and CaMKII–Asp-286 mice (Figure 5A). In the P3 line, which expressed the highest level of CaMKII–Asp-286, we used a second high intensity LTP induction protocol and again found no difference between the transgenic and wild-type mice. These results argue against CaMKII functioning as a simple switch in a pathway for the production of LTP.

A Frequency-Dependent Shift in Synaptic Plasticity

Although not sufficient to induce LTP by itself, CaMKII might instead serve as a regulator of LTP. If this were so, we might expect a shift in the stimulation threshold for the induction of LTP. To test this idea, we stimulated the Schaffer collaterals at three additional frequencies (1, 5, and 10 Hz) and examined the consequent changes in synaptic strength.

As first predicted on theoretical grounds (Bienenstock et al., 1982; Bear et al., 1987) and later confirmed experimentally (Dudek and Bear, 1992), high frequency stimulation leads to LTP, intermediate frequencies produce either no change or a low level of LTP, and low frequencies produce LTD. How is this relationship affected by the expression of the active form of CaMKII? As is the case with rats and guinea pigs (Dudek and Bear, 1993; O'Dell and Kandel, 1994), wild-type mice receiving low frequency (1 Hz) stimulation (900 pulses) show substantial LTD only in the immature animal. With 1 Hz stimulation, hippocampal slices from adult wild-type mice show only a very slight depression (95% ± 2%; n = 7; Figure 6C; all values are percentage of prestimulation synaptic strength ± SEM,

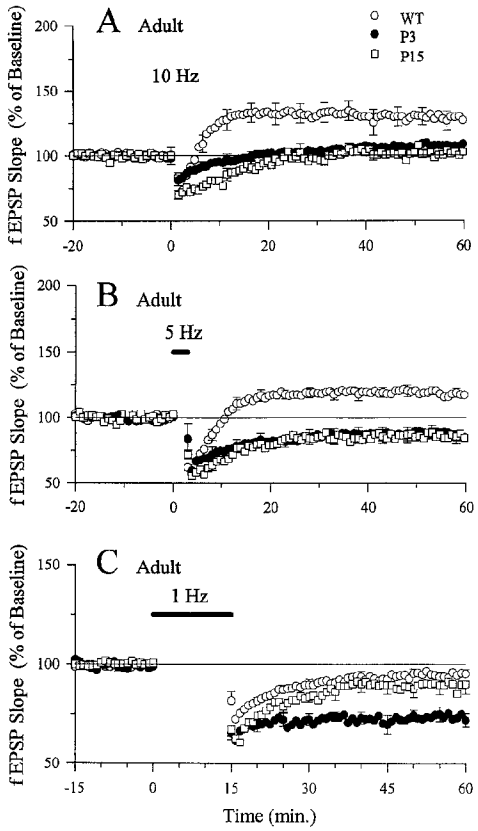

Figure 6. LTP/LTD Produced by Stimulation at 1, 5, and 10 Hz in Adult Mice

Mice ranged in age from 2 to 8 months.
(A) Stimulation at 10 Hz for 1.5 min. Wild type: 129% ± 6% (n = 8); P3: 109% ± 3% (n = 3); P15: 102% ± 2% (n = 4). Wild type versus P3: t(9) = 1.854, $p < .05$; wild type versus P15: t(7) = 2.88, $p < .01$.
(B) Stimulation at 5 Hz for 3 min. Wild type: 117% ± 3% (n = 6); P3: 85% ± 7% (n = 4); P15: 87% ± 5% (n = 3). Wild type versus P3: t(8) = 4.85, $p < 0.01$; wild type versus P15: t(7) = 5.41, $p < .01$.
(C) Stimulation at 1 Hz for 15 min. Wild type: 95% ± 2% (n = 7); P3: 72% ± 3% (n = 3); P15: 90% ± 5% (n = 10). Wild type versus P3: t(8) = 5.59, $p < .01$; wild type versus P15: t(15) = 0.83, not significant.

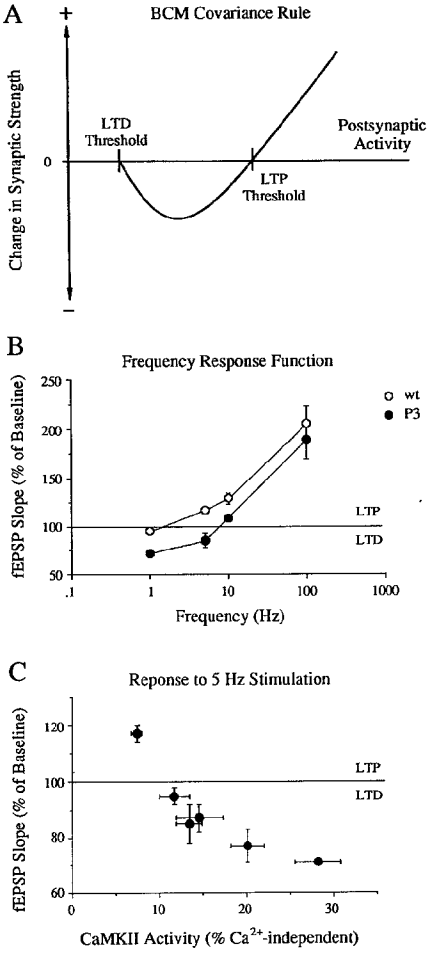

Figure 7. The Frequency–Response Function Shifts with Higher Ca^{2+}-Independent CaMKII Activity

(A) Predicted theoretical relationship between postsynaptic activity and synaptic change (adapted from Bear et al., 1987).
(B) Frequency–response function in transgenic and wild-type mice. The percentage change in synaptic strength from baseline in transgenic and wild-type animals at 1 hr following stimulation at the indicated frequency. Values are mean ± SEM (data from Figure 5A and Figures 6A–6C).
(C) Correlation between 5 Hz plasticity and Ca^{2+}-independent CaMKII activity. Data are for both young and adult wild-type and transgenic animals measured at 1 hr after 5 Hz stimulation (see Figure 6B; Figure 8A). Values are mean ± SEM for both synaptic strength change and percentage of Ca^{2+}-independent CaMKII activity.

measured 1 hr after the onset of stimulation). Stimulation at 5 and 10 Hz (900 pulses) produced LTP to 117% ± 3% (n = 6) and 129% ± 6% (n = 8) of baseline, respectively (Figures 6A and 6B).

In the CaMKII–Asp-286 mice, the same frequencies of stimulation yielded a systematic shift in size and direction of the synaptic change produced by the same presynaptic stimulation. Thus, 10 Hz stimulation, which produced a potentiation in the wild-type mice, produced little change in CaMKII–Asp-286 mice (P3: 109% ± 3% [n = 3]; P15: 102% ± 2% [n = 4]; Figure 6A), and 5 Hz stimulation, which produced a small amount of LTP in wild-type animals, produced a small amount of LTD in the transgenic animals (P3: 85% ± 7% [n = 4]; P15: 87% ± 5% [n = 3]; Figure 6B). Finally, 1 Hz, which produced slight LTD in wild-type animals (95% ± 2%; n = 7), produced substantial LTD in the P3 line (72% ± 3%; n = 3; P3 versus wild type: t(8) = 5.59, $p < .01$). In the P15 line, only a slight enhancement in LTD was observed, and this was not statistically different from that in wild-type animals (90% ± 5%; n = 10; P15 versus wild type: t(15) = 0.83, not significant; Figure 6C). This was the only difference found between the two lines and may be due to the greater level of transgene expression in the P3 line.

That CaMKII could affect LTD, and particularly that it affects it systematically by changing the frequency–response relationship, was unexpected. Since activation of CaMKII is thought to be necessary in the induction of LTP, the transgenic mice in which CaMKII is already partly activated would be expected to undergo LTP more readily. However, at the level of Ca^{2+}-independent CaMKII activity achieved

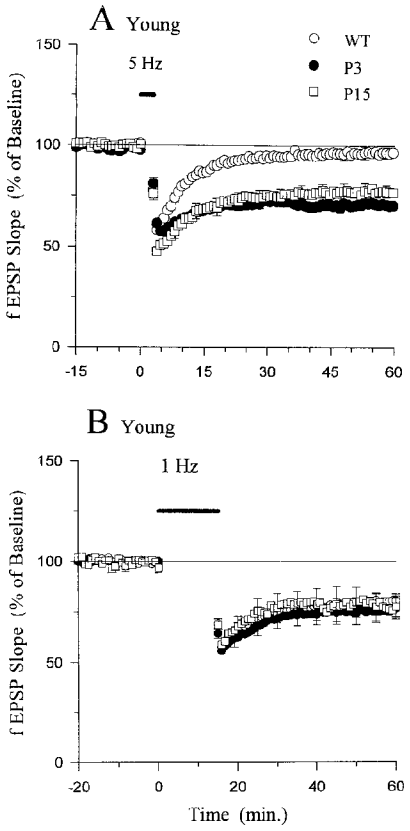

Figure 8. LTD Produced by Stimulation at 1 and 5 Hz in Young Mice

Mice ranged in age from 3 to 4 weeks.

(A) Stimulation at 5 Hz for 3 min. Wild type: 95% ± 3% (n = 6); P3: 71% ± 1% (n = 3); P15: 77% ± 6% (n = 3). Wild type versus P3: $t(7) = 5.75$, $p < .01$; wild type versus P15: $t(7) = 3.13$, $p < .01$.

(B) Stimulation at 1 Hz for 15 min.

in the transgenic animals, we find that the capacity for LTP induction is reduced, while that for LTD is enhanced. Thus, the size and direction of synaptic change seems to be determined by the degree of Ca^{2+}-independent CaMKII activity. When CaMKII is already partially activated, as in the transgenic mice, the frequency–response curve is shifted so as to favor depression (Figure 7B). To explore further the role of Ca^{2+}-independent CaMKII in LTD, we have examined this phenomenon in other contexts.

The Normal Loss of LTD with Age in Wild-Type Animals Parallels Loss of Ca^{2+}-Independent CaMKII Activity

In rats, LTD is robust only in young animals and diminishes with age (Dudek and Bear, 1993). If the ability to produce LTD were related to the degree of autophosphorylation of CaMKII, we might expect that the maturation effect on LTD would be paralleled by changes in CaMKII activity. In fact, decreases in Ca^{2+}-independent CaMKII activity with age have been seen in the rat hippocampus (Molloy and Kennedy, 1991). There is a similar decrease in Ca^{2+}-independent CaMKII activity in the hippocampus as a function of maturity in both wild-type and CaMKII–Asp-286 transgenic mice (see Table 1). Thus, young wild-type mice show an elevated level of Ca^{2+}-independent activity compared with adult wild-type animals (adult, 2–8 months: 7.4 ± 0.6 [n = 10]; young, 3–4 weeks: 11.7 ± 1.7 [n = 9]; $t(17) = 2.5$, $p < 0.025$), and this level is similar to that achieved by the adult transgenics. Young transgenics show the highest levels of Ca^{2+}-independent activity.

If the level of Ca^{2+}-independent CaMKII activity were an important regulator of LTD, we would expect to find enhanced LTD in young wild-type animals as compared with adults, and we would expect the largest LTD in young transgenics. This indeed proved to be the case. LTD produced by 1 Hz stimulation in young wild-type mice is more robust than that produced by the same stimulation in the adult (adult wild type: 95% ± 2% [n = 7]; young wild type: 80% ± 3% [n = 8]; $t(13) = 3.5$, $p < 0.005$). Interestingly, the LTD produced by 1 Hz stimulation in young wild-type animals is similar to that produced in adults from the P3 line of CaMKII–Asp-286 mice (72% ± 3%; n = 3). The young transgenic animals show normal levels of LTD at 1 Hz (Figure 8A). However, the frequency–response curve is again shifted in favor of LTD, so that now, in the young transgenics, 5 Hz stimulation produces robust LTD (P3: 71% ± 1% [n = 3]; P15: 77% ± 6% [n = 3]), whereas in the young wild-type animals, 5 Hz produces only a slight depression in synaptic strength (95% ± 3%; n = 6; Figure 8B).

When the data for young and old, wild-type and transgenic animals are combined, we find that the size and direction of synaptic plasticity produced following 5 Hz stimulation are strongly correlated with the level of Ca^{2+}-independent kinase activity (see Figure 7C). At higher levels of Ca^{2+}-independent CaMKII activity, LTD is enhanced. This suggests it is the developmental decrease in Ca^{2+}-independent CaMKII activity that results in the apparent loss of LTD in the adult wild-type animal. As a corollary, the adult transgenic mice, which show elevated levels of Ca^{2+}-independent CaMKII into adulthood, also maintain some of the phenotypic properties of the immature wild-type animal.

LTP Occludes the Effect of the CaMKII-Asp-286 Mutation

In adult wild-type mice, 5 Hz stimulation of baseline synapses leads to a slight LTP. By contrast, if LTP is first produced by a high frequency train that is known to cause a long-lasting increase in Ca^{2+}-independent CaMKII activity (Fukunaga et al., 1993), then 5 Hz stimulation now produces a depression or depotentiation of the facilitated synapse (Staubli and Lynch, 1990; Fujii et al., 1991; O'Dell and Kandel, 1994). This initially paradoxical finding is consistent with the idea that increased levels of autophosphorylated kinase, in this case produced by synaptic activation, shift the frequency–response function to favor LTD.

To test this idea further, we compared the ability of 5 Hz stimulation to produce synaptic depression following a high frequency train in both transgenic and wild-type animals. Stimulation (5 Hz) of naive synapses revealed a difference between wild-type and transgenic animals, with wild-types showing a potentiation to 117% of baseline and transgenics showing a depression to 86% of baseline (see

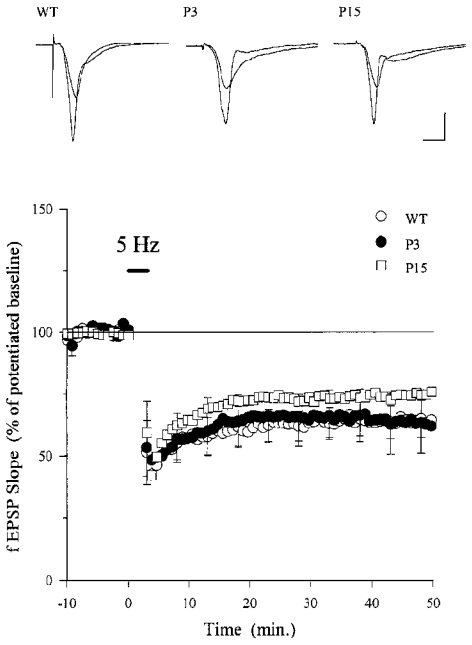

Figure 9. Prior High Frequency Stimulation Occludes the Effect of the CaMKII–Asp-286 Transgene

Slices from transgenic and wild-type animals were first stimulated at 100 Hz, two times for 1 s each, with an interstimulus interval of 10 s. This produced similar levels of LTP in both transgenic and wild-type animals (242.11% ± 19.87% [wild type], 214.58% ± 24.78 [P3], 201.60% ± 19.83% [P15]; wild type versus P3: t(5) = 0.785, not significant; wild type versus P15: t(5) = 1.449, not significant). The baseline was then set at the new potentiated level, and 20 min following the 100 Hz trains, a 5 Hz, 3 min train was delivered. This produced the same level of depression in both transgenic and wild-type animals. The percentage of potentiated baseline 45 min after 5 Hz stimulation was as follows (mean ± SEM): 64% ± 8% (wild type; n = 4 animals, 7 slices), 62% ± 11% (P3; n = 4 animals, 7 slices), 74% ± 3% (P15; n = 3 animals, 7 slices). Wild type versus P3: t(6) = 0.11, not significant; wild type versus P15: t(5) = 1.1, not significant. Traces at the top show field EPSPs recorded in slices from wild-type and transgenic mice just prior to (larger response) and 45 min after (smaller responses) 5 Hz stimulation. Compare with Figure 6B. Bars, 2.5 mV, 4 ms.

Figure 6B). By contrast, following two 100 Hz trains that produced LTP, this difference between wild-type and transgenic animals was occluded so that subsequent 5 Hz stimulation now produced a similar level of depression in both cases (Figure 9). This suggests, as discussed below, that the shift in 5 Hz response following 100 Hz stimulation is due to the elevation in Ca^{2+}-independent CaMKII activity.

Discussion

CaMKII as a Molecular Regulator of the Frequency-Response Function for Synaptic Plasticity

Much current thinking about the mechanisms of associative learning and about parallel associative changes during late stages of development has been guided by a synaptic learning rule postulated by Hebb (1949). Hebb's rule states that the synapse will be strengthened when activity in the pre- and postsynaptic components of a synapse is synchronous. Research over the last several years has made clear that, as stated, this rule is inadequate to account for synaptic modification in the hippocampus and requires modification. A theoretical extension of the Hebb rule called the covariance rule, first proposed by Sejnowski (1977) and later extended by Bienenstock et al. (1982), adds the capacity for synaptic depression to that for facilitation (see Figure 7A). The covariance rule holds that there is a continuum of associative synaptic change that is determined by the relationship between the specific level of postsynaptic depolarization paired with presynaptic activity. Strong postsynaptic depolarization leads to synaptic potentiation, weak depolarization to depression. Artola and Singer (1993) have elaborated this idea further by suggesting that different levels of depolarization lead to different levels of Ca^{2+} influx into the postsynaptic cell (Artola et al., 1990), and that the Ca^{2+} influx is critical for both depression and potentiation. Our data and those of Dudek and Bear (1992) provide independent support for the continuity between LTP and LTD and suggest specifically that a key determinant of postsynaptic depolarization and consequent Ca^{2+} influx is the frequency of presynaptic stimulation. Different frequencies produce different patterns of postsynaptic depolarization and, presumably, different patterns of NMDA receptor–mediated Ca^{2+} influx, resulting in a systematic relationship between presynaptic firing frequency, postsynaptic depolarization, Ca^{2+} influx, and synaptic strength. For the same number of presynaptic pulses, a high frequency of presynaptic firing leads to greater temporal summation of the individual EPSPs, and thus a greater overall postsynaptic depolarization. As a result, there will be greater Ca^{2+} influx, which favors the generation of LTP. Lower frequencies produce more modest postsynaptic depolarization and a lower level of Ca^{2+} influx. This seems to favor the generation of LTD.

Our data indicate that the frequency–response function for the production of LTP and LTD is closely regulated by development and prior synaptic activity. Moreover, our results show that CaMKII is an important molecular regulator of this function. Thus, in mice expressing elevated levels of Ca^{2+}-independent CaMKII, the entire frequency–response curve is shifted systematically to favor LTD. This yields the result that, at moderate frequencies of stimulation, LTP is lost in the transgenics and LTD is enhanced.

How Does the Ca^{2+}-Independent CaMKII Produce Its Action?

According to the simple switch hypothesis of LTP, CaMKII activation alone is sufficient to induce and maintain synaptic potentiation, perhaps through the phosphorylation of postsynaptic glutamate receptors (McGlade-McCulloh et al., 1993; Tan et al., 1994). Our results provide a view of the function of CaMKII that is somewhat different from the conventional switch hypothesis.

In the CaMKII–Asp-286 mice, there is no alteration in the total amount of LTP obtained at high frequencies, but there is a systematic shift to favor LTD at medium to low frequencies. Thus, our results suggest a direct role for

CaMKII in the production of LTD. The observation that mice with a targeted disruption of CaMKIIα lack LTD supports this interpretation (Stevens et al., 1994). How can the apparent importance of CaMKII for LTD be reconciled with the genetic and pharmacological evidence suggesting a critical role for CaMKII in LTP? Our data are consistent with the two possibilities outlined in Figure 10.

In the first model, it is not the enzymatic activity of the autophosphorylated form of CaMKII that is critical but the 100-fold increased affinity of the autophosphorylated CaMKII subunit for Ca^{2+}/calmodulin (Meyer et al., 1992; Hanson et al., 1994). Here, CaMKII would function as a regulatable Ca^{2+}/calmodulin sink that is sensitive to the prior history of Ca^{2+} influx into the dendritic spine in which it is located (Figure 10A). According to this view, both LTP and LTD require the action of Ca^{2+}/calmodulin, with low levels of free Ca^{2+}/calmodulin leading to LTD and high Ca^{2+}/calmodulin leading to LTP. In this case, CaMKII enzyme activity is not necessary for the induction of either LTP or LTD. However, the amount of autophosphorylated CaMKII regulates the balance between the two processes by regulating the levels of free Ca^{2+}/calmodulin in the dendritic spine. The dissociation constant for calmodulin from CaMKII increases from about 0.2 s in the unphosphorylated state to about 20 s for CaMKII that is autophosphorylated at Thr-286. Thus, at higher levels of autophosphorylated CaMKII, the same size Ca^{2+} signal would produce a smaller increase of free Ca^{2+}/calmodulin, owing to the sequestration of a larger fraction of the Ca^{2+}/calmodulin by the high affinity autophosphorylated form of the kinase. This would in turn lead to a systematic shift in favor of LTD at all submaximal levels of Ca^{2+} influx (i.e., at low frequencies of stimulation). This effect of the autophosphorylated kinase should be overcome at saturating levels of Ca^{2+}/calmodulin, as might be achieved by high frequency stimulation.

This model depends on the ability of CaMKII phosphorylated at Thr-286 to bind tightly to Ca^{2+}/calmodulin. It is clear that Thr-286 phosphorylation is sufficient to convert the enzyme to the high affinity form (Hanson et al., 1994); however, the ability of the CaMKII–Asp-286 mutant form of the kinase to mimic autophosphorylation in this case, and thereby trap Ca^{2+}/calmodulin, has not been tested. The model in Figure 10A is directly testable by expression of a form of CaMKII containing the Thr-286→Asp mutation as well as a second site mutation (Lys-42→Met), which knocks out enzymatic activity. This should allow the dissociation of the effects of the Thr-286→Asp mutation on enzyme activity from the effect on Ca^{2+}/calmodulin trapping.

According to the second view, CaMKII activation is necessary for both LTP and LTD, but is the rate limiting step only for LTD (Figure 10B). While strong evidence exists for the involvement of CaMKII in LTP, there is equally strong evidence for the involvement, in the induction process, of other kinases such as tyrosine kinases and protein kinase C (O'Dell et al., 1991; Grant et al., 1992; Abeliovich et al,. 1993). If CaMKII activation were necessary but not sufficient for the generation of LTP, then expression of the activated form of the enzyme should not produce LTP and therefore should not alter the level of LTP obtained at high frequencies. If CaMKII activation were not the rate-limiting step in LTP induction, then no enhancement in the ability to induce LTP should be observed in the transgenic mice. However, CaMKII activation appears also to be necessary for LTD induction, perhaps along with the activation of other molecules such as protein phosphatases. If CaMKII activation were the rate-limiting step in LTD induction, then the CaMKII–Asp-286 mice, in which CaMKII is already partially active, would show an enhanced capacity to undergo LTD.

The two models we present postulate that the effect of the CaMKII–Asp-286 transgene is postsynaptic. However, since the transgene is also expressed in the presynaptic CA3 neurons, it may exert its effects on the frequency–response function presynaptically. In fact, although the induction of both LTP and LTD occurs postsynaptically and requires Ca^{2+} influx into the postsynaptic neuron, both

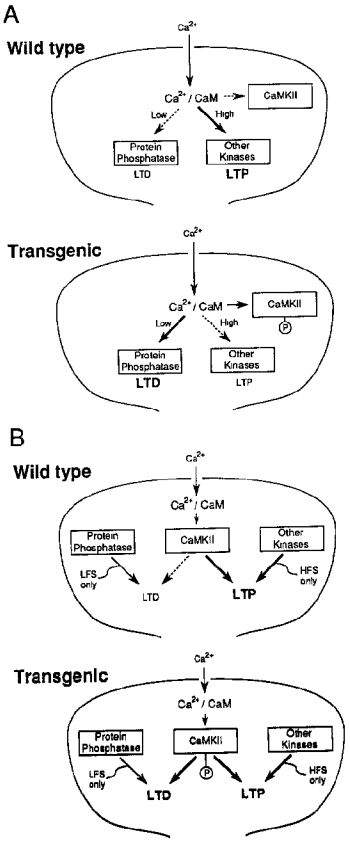

Figure 10. Possible Mechanisms for CaMKII Regulation of LTP and LTD

Both models assume that the level of Ca^{2+} influx increases with increasing frequency of stimulation.

(A) Calmodulin trapping model. In the transgenic animals, the greater level of autophosphorylated CaMKII binds a greater amount of Ca^{2+}/calmodulin, shifting the balance toward LTD at all but the highest levels of Ca^{2+} influx.

(B) Enzyme activity model. In the transgenic animals, the enzyme is already partially activated such that, at low levels of Ca^{2+} influx, greater enzyme activity, which is necessary for LTD, is achieved.

CaM, calmodulin; HFS, high frequency stimulation; LFS, low frequency stimulation; P, phosphorylation at Thr-286.

processes appear to be maintained presynaptically (Bolshakov and Siegelbaum, 1994; Stevens and Wang, 1994). Moreover, during prolonged stimulation, significant Ca^{2+} elevation occurs in the presynaptic terminal, and it has been suggested that presynaptic kinase activity is important for the maintenance of LTP. The two alternative biochemical mechanisms outlined in Figure 10 could therefore also apply to the presynaptic terminal.

In contrast to our result, Pettit et al. (1994) have shown that infection of hippocampal slices with a vaccinia virus expressing a truncated Ca^{2+}-independent form of CaMKII enhances synaptic responses and blocks LTP. It is unclear why we did not see an enhanced synaptic response or block of LTP in the CaMKII–Asp-286 transgenic mice, since we achieved a 5-fold greater increase in Ca^{2+}-independent CaMKII activity than did Pettit et al. One difference was that they expressed a truncated monomeric form of CaMKII in hippocampal slices using a vaccinia virus vector. The truncated form of the enzyme does not contain the domains necessary for proper assembly of the enzyme into multimers or for the dendritic localization of mRNA. This form of the enzyme may therefore be aberrantly localized within the neuron. Also, it is possible that some synergism between the CaMKII expression and the normal progression of the vaccinia virus life cycle, which will eventually kill the neuron, leads to the LTP block. Alternatively, it may be that in the transgenic animals some compensatory change occurs during development that restores the capacity for LTP even at high levels of Ca^{2+}-independent CaMKII activity.

A Role for CaMKII in Depotentiation

Prior theoretical and experimental work has suggested that the frequency–response function is not fixed, but can be modified by prior synaptic input (Bienenstock et al., 1982; Bear et al., 1987; Staubli and Lynch, 1990; Fujii et al., 1991; O'Dell and Kandel, 1994). Bienenstock et al. specifically suggested that the frequency–response function is regulated by the time-averaged activity of the neuron. Our data suggest that CaMKII serves as the sensor of the level of neuronal activity and in turn controls the frequency–response function. When a synapse is activated at medium to high frequencies, three changes occur: Ca^{2-}-independent CaMKII activity is increased, some LTP is produced, and the frequency–response function is shifted to favor LTD. Our data suggest that the elevated Ca^{2+}-independent kinase causes the shift in the frequency–response function to favor LTD. Thus, at a previously stimulated synapse, future stimuli will be less likely to produce LTP and more likely to produce LTD. In this way, CaMKII can serve as a molecular regulator of the dynamic range of synaptic strength.

An example of the role of CaMKII as a sensor of neuronal activity is illustrated in our study of depotentiation. In mature wild-type animals, stimulation of naive synapses at 5 Hz in hippocampal area CA1 leads to a small potentiation above baseline. If LTP is first induced by high frequency stimulation, subsequent 5 Hz stimulation produces a strong depression. The CaMKII–Asp-286 mice differ from wild types in that stimulation of the naive synapse at 5 Hz produces a depression. However, following high frequency stimulation, the responses of transgenic and wild-type slices to 5 Hz are indistinguishable. Thus, prior synaptic stimulation can occlude the effect of the CaMKII–Asp-286 transgene. These data, in addition to providing a test of the importance of the Ca^{2+}-independent CaMKII in shifting the frequency–response function, also serve as important controls for the secondary consequences of the transgene.

In the generation of transgenic mice, it is always possible that the observed phenotype results from a secondary effect of transgene expression. For example, the observed difference in response to 5 Hz stimulation in the CaMKII–Asp-286 mice as compared with wild types could result from a subtle change in development or from effects on neuronal processes not directly related to LTP. However, the occlusion of the phenotypic difference between wild-type and transgenic animals by LTP-inducing stimuli suggests that this is not the case. The high frequency stimulation would not be expected to reverse a developmental difference in the transgenic animals or to alter signaling pathways unrelated to LTP. However, this result would be expected if the high frequency 100 Hz stimulation produced a saturating level of Ca^{2+}-independent CaMKII activity, even in the wild-type animal. In this case, the CaMKII in the transgenic and wild-type animals would be stimulated to the same level, or beyond some threshold level of Ca^{2+}-independent activity, and thus show the same frequency–response function. Alternatively, if the shift in 5 Hz response caused by prior 100 Hz stimulation were mediated by something other than changes in CaMKII activity, the effect of 100 Hz stimulation should be additive with the effect of CaMKII–Asp-286 expression, and the transgenic mice should continue to show a larger 5 Hz depression than wild-type animals, even following 100 Hz stimulation.

Changes in CaMKII Activity in Maturation May Contribute to the Closure of the Critical Period

The amount of Ca^{2+}-independent CaMKII activity in hippocampal extracts normally decreases during development between 3 and 8 weeks of age (see Table 1) (Molloy and Kennedy, 1991). During this time, there is also a corresponding decrease in the ability of low frequency stimulation to produce LTD. In the CaMKII–Asp-286 mice, where the Ca^{2+}-independent kinase activity in adults is maintained at an elevated level similar to that of a 3-week-old wild-type animal, LTD produced by low frequency stimulation is enhanced. These results suggest that hippocampal synapses normally undergo a developmental shift in the frequency–response function that is mediated, at least in part, by a corresponding change in CaMKII activity. In the CaMKII–Asp-286 mice, the CaMKII activity due to the expression of the transgene maintains the synapses at the juvenile setting.

While the amount of LTD obtained at 5 Hz is clearly correlated with Ca^{2+}-independent CaMKII levels (see Figure 7B), it is unclear whether the quantitative level of LTD attainable is regulated by CaMKII. At 1 Hz, only the P3 line of transgenics shows quantitatively enhanced LTD in

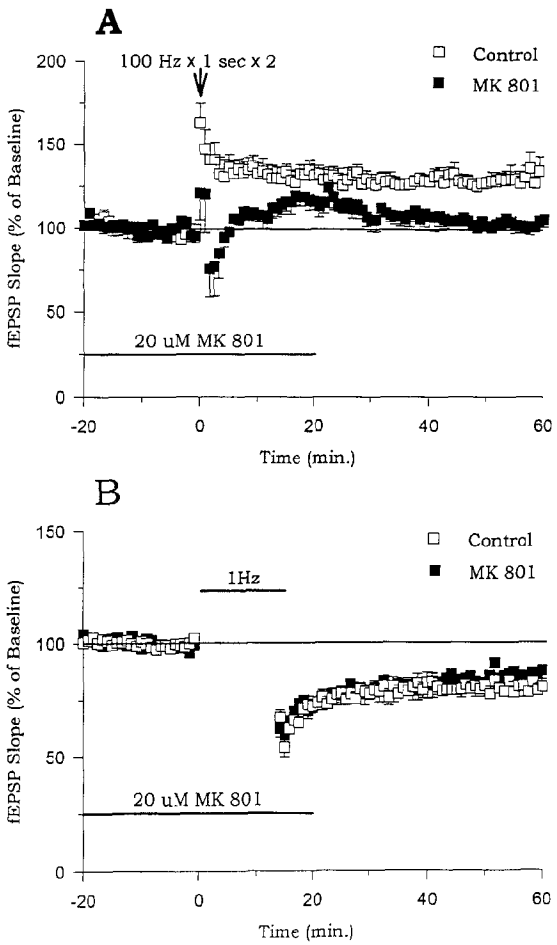

Figure 11. MK801 Blocks LTP but Not LTD

(A) Hippocampal slices from 2- to 3-week-old wild-type mice were stimulated at 100 Hz two times, 1 s each, with an interstimulus interval of 20 s. Stimulation was at 50% of the maximal EPSP. Application of 20 μM MK801 as indicated completely blocked LTP (control: 129% ± 5% [n = 5]; MK801: 103% ± 3% [n = 3]; $t(6) = 3.68$, $p < .02$).

(B) Stimulation at 1 Hz for 15 min produced LTD that was not blocked by 20 μM MK801 (control: 80% ± 4% [n = 7]; MK801: 83% ± 5% [n = 8]; $t(13) = .377$, not significant). Slices in 20 μM MK801 showed significant depression at 1 hr poststimulus onset compared with baseline ($t(7) = 3.44$, $p < .02$).

the adult animal. This line expresses a greater level of mutant transgene than the P15 line, even though the Ca^{2+}-independent activity measured in adult hippocampal extracts is equal in the P3 and P15 lines. This suggests that there may be some compensatory change in a protein phosphatase activity in the P3 line. It may be that the absolute level of LTD obtainable is mediated by the level of phosphatase expressed, while the frequency threshold is mediated by the level of Ca^{2+}-independent CaMKII activity.

In young animals the capacity for LTD is enhanced and is in fact present in the hippocampus prior to the appearance of LTP (Müller et al., 1989). What might be the function of the enhanced ability to depress synapses through activity in young animals? During neuronal development the major projections between brain regions are set up using extracellular cues for axonal growth and guidance. In a particular region, such as the lateral geniculate or striate cortex, the initial axonal arborization is diffuse and only later becomes segregated through an activity-dependent process thought to involve a mechanism similar to that of LTP (see Goodman and Shatz, 1993, for review). When this normal developmental fine tuning is blocked with 2-amino-5-phosphonovaleric acid (APV) or tetrodotoxin, synaptic connections that would normally be lost during maturation to adulthood are maintained (Stryker and Harris, 1986; Shatz and Stryker, 1988; Bear et al., 1990). This suggests that a mechanism of selective activity-dependent synaptic depression such as LTD may be an important mechanism by which the inappropriate connections in young animals are lost. Both LTD and LTP require NMDA receptor activation, and both are blocked by APV, which also blocks synapse segregation. However, Cline and Constantine-Paton (1990) have found that segregation of axonal arbors in the frog tectum is not blocked by MK801, an open-channel blocker of the NMDA receptor. We therefore examined the effect of MK801 on hippocampal plasticity and found that, though it blocks LTP, it does not affect LTD produced at low frequencies such as 1 Hz (Figure 11). This supports the idea that a LTD-like phenomenon may be the predominant mechanism for activity-dependent synaptic segregation. If this interpretation is correct, the decrease of Ca^{2+}-independent CaMKII activity may signal the end of a critical period of development by reducing the capacity for LTD. One prediction of this model is that in the CaMKII–Asp-286 mice the critical period for developmental fine tuning should be extended into adulthood.

The CaMKII–Asp-286 mice provide a useful model for the study of the role of synaptic change produced at different frequencies of neuronal firing. The shift in the transgenics is pronounced in the range of the θ rhythm (4–12 Hz), at which learning-related synaptic plasticity in the hippocampus is thought to be produced in freely behaving animals. In the accompanying paper (Bach et al., 1995 [this issue of Cell]), we explore the effects of this shift in θ frequency synaptic plasticity on hippocampally mediated learning and memory tasks.

Experimental Procedures

Transgene Construction

The full-length mouse CaMKIIα cDNA and a genomic clone containing the 5′ portion of the CaMKIIα coding region, as well as 8.5 kb of upstream DNA, were isolated from a mouse brain (C57BL6) cDNA library and a mouse spleen (C57BL6) cosmid library, respectively, using a rat cDNA probe (kindly provided by N. Sahyoun; Sunyer and Sahyoun, 1990). The Thr-286→Asp mutation was introduced into the cDNA clone between an XmnI and SpeI site using a synthetic fragment composed of the following two oligonucleotides: 5′-CACAGACAGGAGGACGTCGACTGCCTGAAGA-3′ and 5′-TCTTCAGGCAGTCGACGTCCTCCTGTCTGTGCATG-3′. The 3 bp change from the wild-type sequence introduces an AatII site into the Thr-286→Asp mutant cDNA. The poly(A) signal from bovine growth hormone (obtained from plasmid pRc/CMV, Invitrogen) was placed 3′ to the cDNA. The cDNA was fused in phase to the corresponding exon in the genomic clone using an EagI site present in both. Cloning junctions were verified by DNA sequencing. The 45 kb insert (see Figure 1A) was removed from the vector by NotI digestion and used for the generation of transgenic mice.

Transgenic Mice

Transgenic mice were generated by DNX, Inc., using C57BL6/SJL F2 hybrid embryos for microinjection. Founders were backcrossed onto a C57BL6 background. Mice were screened for the transgene by Southern blot or PCR for routine typing. Animals were maintained and bred under standard conditions.

Northern Blot and PCR

The hippocampus was removed from transgenic or wild-type mice, and RNA was purified using the guanidium thiocyanate method. For Northern blots, 20 μg of total hippocampal RNA was used. Filters were hybridized to an [α-^{32}P]dATP-tailed oligonucleotide probe specific for the Thr-286→Asp mutation (5′-CTTCAGGCAGTCGACGTCCTCCTG-TCTGTG-3′). Hybridization was at 42°C overnight in a solution containing 50% formamide, 10% dextran sulfate, 25 mM HEPES, 1 mM EDTA, 6× Denhardt's, 240 mM NaCl, 100 μg/ml denatured salmon sperm DNA, and 100 μg/ml poly(dA). Filter was washed once for 10 min in 2× SSC at room temperature and three times for 10 min each in 0.2× SSC at 60°C and exposed to film for 1 week.

For RT–PCR, 100 ng of total hippocampal RNA was amplified in a 100 μl reaction with rTth DNA polymerase (Perkin Elmer) as recommended by the manufacturer. Amplification was for 40 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The following oligonucleotides were used as primers: 5′-AGATGCTGACCATCAACCCA-3′ and 5′-CTCTGGTTCAAAGGCTGTCA-3′. These produce a 401 bp fragment containing the site of the Thr-286→Asp mutation. The PCR reaction was done in the presence of 1 μCi of [α-^{32}P]dCTP, and the amplified fragment was digested with AatII. The AatII site is present only in the mutant transcripts, and digestion leads to the production of 294 and 107 bp fragments. The digested sample was run on a 2% agarose gel. The gel was fixed, dried, and exposed to film. The expression of the mutant transgene relative to the wild type was estimated by densitometric comparison of the respective bands on the autoradiogram.

In Situ Hybridization

Mice were killed by cervical dislocation, and the brains were dissected and rapidly frozen in mounting medium. Cryostat sections (20 μm) were taken, postfixed for 10 min in 4% paraformaldehyde in PBS (pH 7.3), dehydrated, and stored frozen at −70°C until use. The slices were hybridized to the Asp-286-specific oligonucleotide probe or to a probe specific for the wild-type mouse CaMKIIα gene (5′-CTCAGGC-AGTCCACGGTCTCCTGTCTGTG-3′). The probes were labeled by 3′ poly(A) tailing using [α-^{35}S]thio-dATP and terminal transferase to a specific activity of 0.5 × 10^9 to 1 × 10^9 cpm/μg. Hybridization was overnight at 42°C in a solution containing 50% formamide, 10% dextran sulfate, 25 mM HEPES (pH 7.0), 600 mM NaCl, 100 mM DTT, 1× Denhardt's, 200 μg/ml denatured salmon sperm DNA, 200 μg/ml poly(dA), and 10^7 cpm/ml oligonucleotide probe. Slides were washed two times for 10 min each in 2× SSC at room temperature and two times for 60 min each in 0.2× SSC at 65°C, dried, and exposed to film for 3 weeks (mutant probe) or 4 days (wild-type probe).

Electrophysiology

Transverse slices (400 μm thick) of mouse hippocampus were prepared using standard techniques (see Grant et al., 1992) and maintained in an interface-type recording chamber perfused (1–3 ml/min) with a mouse artificial cerebrospinal fluid consisting of 124 mM NaCl, 4.4 mM KCl, 12.0 mM Na_2PO_4, 25 mM Na_2HCO_3, 2.0 mM $CaCl_2$, 2.0 mM $MgSO_4$, and 10 mM glucose, gassed with 95% O_2 and 5% CO_2. All experiments were done at 30°C and began after allowing the tissue to recover for at least 60 min following slice preparation.

EPSPs were elicited once every 50 s (0.02 Hz) using nichrome or tungsten wire bipolar stimulation electrodes (0.01–0.02 ms duration pulses) placed in stratum radiatum of the CA1 region. The resulting potentials were monitored using low resistance glass microelectrodes (5–10 MΩ, filled with artificial cerebrospinal fluid) also placed in stratum radiatum of CA1. In each experiment, we first determined the maximal field EPSP amplitude by gradually increasing the stimulation intensity until the EPSP amplitude reached a saturating level. The stimulation intensity was then decreased so as to evoke a response that was ~50% of the maximal EPSP amplitude. In all experiments baseline synaptic transmission was monitored for 15–20 min before delivering either high or low frequency stimulation. In general, LTP was induced using two trains of 100 Hz stimulation (each 1 s in duration; intertrain interval = 10 s). However, in one series of experiments (see Figure 5B), baseline EPSPs were evoked using a stimulation intensity sufficient to evoke EPSPs that were 25% of the maximum, and LTP was induced using two trains of 100 Hz stimulation (1 s duration) separated by 20 s and delivered at an intensity sufficient to evoke EPSPs that were 75% of the maximum. In the majority of the electrophysiological experiments, the experimenter was blind to the genotype of the animal. For all statistical comparisons, the n used was the number of animals rather than number of slices.

CaMKII Enzyme Assays

Hippocampal slices were prepared in the same manner as those used for electrophysiology and then incubated for 1 hr at 30°C in an oxygenated chamber. Extracts were prepared from the entire slice and enzyme assayed essentially as described by Ocorr and Schulman (1991). Slices were frozen rapidly in liquid nitrogen and homogenized in a buffer containing 20 mM Tris–HCl (pH 7.5), 0.5 mM EGTA, 0.5 mM DTA, 2 mM leupeptin, 0.4 mM DTT, and 0.1 mM PMSF, either with or without phosphatase inhibitors (0.4 mM molybdate, 10 mM sodium pyrophosphate). Protein levels were assayed using the micro BCA Kit (Pierce) with BSA as standard. Samples without phosphatase inhibitors were incubated for 15 min at room temperature before enzyme assay. Enzyme reaction mix consisted of 50 mM PIPES (pH 7.0), 10 mM $MgCl_2$, 100 μg/ml BSA, 200 μg/ml leupeptin, 0.4 mM DTT, 0.6 mM EGTA, 0.2 mM EDTA, 200 μM ATP, 100 μCi/ml [α-^{32}P]ATP, and 20 μM autocamtide-2, with either 1 mM $CaCl_2$ and 5 μg/ml calmodulin ($+Ca^{2+}$) or 2 mM EGTA ($-Ca^{2+}$). Enzyme reactions were carried out at 30°C for 1 min in a final volume of 50 μl. The reaction was initiated by the addition of 0.5–2 μg of hippocampal extract and terminated by the addition of an equal volume of 10% ice-cold TCA. Protein was pelleted and the supernatant was spotted onto Whatman P81 filter paper disks and washed three times for 10 min each with water. The level of incorporated ^{32}P was determined by scintillation counting.

Acknowledgments

We thank Richard Axel and Tom Jessell for critically reading this manuscript. We also thank Katrina Podsypanina for maintaining and genotyping the mice, Sarah Mack and Chuck Lam for helping with the figures, Harriet Ayers and Andrew Krawetz for typing the manuscript, and Mohamed Osman for helping with animal care. This research was supported by the Howard Hughes Medical Institute, the Charles A. Dana Foundation, and NIMH grant MH50733. T. J. O. was also supported by a Klingenstein Fellowship in the Neurosciences.

Received March 6, 1995; revised April 13, 1995.

References

Abeliovich, A., Chen, C., Goda, Y., Silva, A. J., Stevens, C. F., and Tonegawa, S. (1993). Modified hippocampal long-term potentiation in PKCγ-mutant mice. Cell *75*, 1253–1262.

Artola, A., and Singer, W. (1993). Long-term depression of excitatory synaptic transmission and its relationship to long-term potentiation. Trends Neurosci. *16*, 480–488.

Artola, A., Bröcher, S., and Singer, W. (1990). Different voltage-dependent thresholds for inducing long-term depression and long-term potentiation in slices of rat visual cortex. Nature *347*, 69–72.

Bach, M. E., Hawkins, R. D., Osman, M., Kandel, E. R., and Mayford, M. (1995). Impairment of spatial but not contextual memory in CaMKII mutant mice with a selective loss of hippocampal LTP in the range of the θ frequency. Cell, this issue.

Bading, H., Ginty, D. D., and Greenberg, M. E. (1993). Regulation of gene expression in hippocampoal neurons by distinct calcium signaling pathways. Science *260*, 181–186.

Bear, M. F., and Malenka, R. C. (1994). Synaptic plasticity: LTP and LTD. Curr. Opin. Neurobiol. *4*, 389–399.

Bear, M. F., Cooper, L. N., and Ebner, F. F. (1987). A physiological basis for a theory of synapse modification. Science *237*, 42–48.

Bear, M. F., Kleinschmidt, A., Gu, Q., and Singer, W. (1990). Disruption

of experience-dependent synaptic modifications in striate cortex by infusion of an NMDA receptor antagonist. J. Neurosci. *10*, 909–925.

Bienenstock, E., Cooper, L., and Munro, P. (1982). Theory for the development of neuron selectivity: orientation specificity and binocular interaction in visual cortex. J. Neurosci. *2*, 32–48.

Bliss, T. V. P., and Collingridge, G. L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. Nature *361*, 31–39.

Bolshakov, V. Y., and Siegelbaum, S. A. (1994). Postsynaptic induction and presynaptic expression of hippocampal long-term depression. Science *264*, 1148–1152.

Cline, H. T., and Constantine-Paton, M. (1990). NMDA receptor agonist and antagonists alter retinal ganglion cell arbor structure in the developing frog retinotectal projection. J. Neurosci. *10*, 1197–1216.

Dudek, S. M., and Bear, M. F. (1992). Homosynaptic long-term depression in area CA1 of hippocampus and effects of *N*-methyl-D-aspartate receptor blockade. Proc. Natl. Acad. Sci. USA *89*, 4363–4367.

Dudek, S. M., and Bear, M. F. (1993). Bidirectional long-term modification of synaptic effectiveness in the adult and immature hippocampus. J. Neurosci. *13*, 2910–2918.

Fong, Y.-L., Taylor, W. L., Means, A. R., and Soderling, T. R. (1989). Studies of the regulatory mechanism of Ca^{2+}/calmodulin-dependent protein kinase II: mutation of threonine 286 to alanine and aspartate. J. Biol. Chem. *264*, 16759–16763.

Fujii, S., Saito, K., Miyakawa, H., Ito, K., and Kato, H. (1991). Reversal of long-term potentiation (depotentiation) induced by tetanus stimulation of the input to CA1 neurons of guinea pig hippocampal slices. Brain Res. *555*, 112–122.

Fukunaga, K., Stoppin, L., Miyamoto, E., and Muller, D. (1993). Long-term potentiation is associated with an increased activity of Ca^{2+}/calmodulin-dependent protein kinase II. J. Biol. Chem. *268*, 7863–7867.

Goodman, C. S., and Shatz, C. J. (1993). Developmental mechanisms that generate precise patterns of neuronal connectivity. Cell *72*/Neuron *10* (Suppl.), 77–98.

Grant, S. G. N., O'Dell, T. J., Karl, K. A., Stein, P., Soriano, P., and Kandel, E. R. (1992). Impaired long-term potentiation, spatial learning, and hippocampal development in *fyn* mutant mice. Science *258*, 1903–1910.

Hanson, P. I., and Schulman, H. (1992). Neuronal Ca^{2+}/calmodulin-dependent protein kinases. Annu. Rev. Biochem. *61*, 559–601.

Hanson, P. I., Meyer, T., Stryer, L., and Schulman, H. (1994). Dual role of calmodulin in autophosphorylation of multifunctional CaM kinase may underlie decoding of calcium signals. Neuron *12*, 943–956.

Hebb, D. O. (1949). The Organization of Behaviour (New York: Wiley).

Kamiya, H., and Zucker, R. S. (1994). Residual Ca^{2+} and short-term synaptic plasticity. Nature *371*, 603–606.

Lin, J.-W., Sugimori, M., Llinás, R. R., McGuiness, T., and Greengard, P. (1990). Effects of synapsin I and calcium/calmodulin-dependent protein kinase II on spontaneous neurotransmitter release in the squid giant synapse. Proc. Natl. Acad. Sci. USA *87*, 8257–8261.

Lisman, J. (1989). A mechanism for the Hebb and the anti-Hebb processes underlying learning and memory. Proc. Natl. Acad. Sci. USA *86*, 9574–9578.

Lisman, J. (1994). The CaM kinase II hypothesis for the storage of synaptic memory. Trends Neurosci. *17*, 406–412.

Llinás, R., McGuinness, T. L., Leonard, C. S., Sugimori, M., and Greengard, P. (1985). Intraterminal injection of synapsin I or calcium/calmodulin-dependent protein kinase II alters neurotransmitter release at the squid giant synapse. Proc. Natl. Acad. Sci. USA *82*, 3035–3039.

Lou, L. L., and Schulman, H. (1989). Distinct autophosphorylation sites sequentially produce autonomy and inhibition of the multifunctional Ca^{2+}/calmodulin-dependent protein kinase. J. Neurosci. *9*, 2020–2032.

Lynch, G., Larson, J., Kelso, S., Barrionuevo, G., and Schottler, F. (1983). Intracellular injections of EGTA block induction of hippocampal long-term potentiation. Nature *305*, 719–721.

Malenka, R. C., Kauer, J. A., Perkel, D. J., Mauk, M. D., Kelly, P. T., Nicoll, R. A., and Waxham, M. N. (1989). An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. Nature *340*, 554–557.

Malenka, R. C., Lancaster, B., and Zucker, R. S. (1992). Temporal limits on the rise in postsynaptic calcium required for the induction of long-term potentiation. Neuron *9*, 121–128.

Malinow, R., Schulman, H., and Tsien, R. W. (1989). Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. Science *245*, 862–866.

McGlade-McCulloh, E., Yamamoto, H., Tan, S.-E., Brickey, D. A., and Soderling, T. R. (1993). Phosphorylation and regulation of glutamate receptors by calcium/calmodulin-dependent protein kinase II. Nature *362*, 640–642.

McGuinness, T. L., Lai, Y., and Greengard, P. (1985). Ca^{2+}/calmodulin-dependent protein kinase II. J. Biol. Chem. *260*, 1696–1704.

Meyer, T., Hanson, P. I., Stryer, L., and Schulman, H. (1992). Calmodulin trapping by calcium-calmodulin-dependent protein kinase. Science *256*, 1199–1202.

Miller, S. G., and Kennedy, M. B. (1985). Distinct forebrain and cerebellar isozymes of type II Ca^{2+}/calmodulin-dependent protein kinase associate differently with the postsynaptic density fraction. J. Biol. Chem. *260*, 9039–9046.

Miller, S. G., and Kennedy, M. B. (1986). Regulation of brain type II Ca^{2+}/calmodulin-dependent protein kinase by autophosphorylation: a Ca^{2+}-triggered molecular switch. Cell *44*, 861–870.

Miller, S. G., Patton, B. L., and Kennedy, M. B. (1988). Sequences of autophosphorylation sites in neuronal type II CaM kinase that control Ca^{2+}-independent activity. Neuron *1*, 593–604.

Molloy, S. S., and Kennedy, M. B. (1991). Autophosphorylation of type II Ca^{2+}/calmodulin-dependent protein kinase in cultures of postnatal rat hippocampal slices. Proc. Natl. Acad. Sci. USA *88*, 4756–4760.

Mulkey, R. M., and Malenka, R. C. (1992). Mechanisms underlying induction of homosynaptic long-term depression in area CA1 of the hippocampus. Neuron *9*, 967–975.

Mulkey, R. M., Herron, C. E., and Malenka, R. C. (1993). An essential role for protein phosphatases in hippocampal long-term depression. Science *261*, 1051–1058.

Mulkey, R. M., Endo, S., Shenolikar, S., and Malenka, R. C. (1994). Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. Nature *369*, 486–488.

Müller, D., Oliver, M., and Lynch, G. (1989). Developmental changes in synaptic properties in hippocampus of neonatal rats. Dev. Brain Res. *49*, 105–114.

Nichols, R. A., Sihra, T. S., Czernik, A. J., Nairn, A. C., and Greengard, P. (1990). Calcium/calmodulin-dependent protein kinase II decreases glutamate and noradrenaline release from synaptosomes. Nature *343*, 647–651.

Ocorr, K. A., and Schulman, H. (1991). Activation of multifunctional Ca^{2+}/calmodulin-dependent kinase in intact hippocampal slices. Neuron *6*, 907–914.

O'Dell, T. J., and Kandel, E. R. (1994). Low-frequency stimulation erases LTP through an NMDA receptor-mediated activation of protein phosphatases. Learn. Mem. *1*, 129–139.

O'Dell, T. J., Kandel, E. R., and Grant, S. G. N. (1991). Long-term potentiation in the hippocampus is blocked by tyrosine kinase inhibitors. Nature *353*, 558–560.

Pettit, D. L., Perlman, S., and Malinow, R. (1994). Potentiated transmission and prevention of further LTP by increased CaMKII activity in postsynaptic hippocampal slice neurons. Science *266*, 1881–1885.

Schworer, C. M., Colbran, R. J., Keefer, J. R., and Soderling, T. R. (1988). Ca^{2+}/calmodulin-dependent protein kinase II: identification of a regulatory autophosphorylation site adjacent to the inhibitory and calmodulin-binding domains. J. Biol. Chem. *263*, 13486–13489.

Sejnowski, T. J. (1977). Storing covariance with nonlinearly interacting neurons. J. Math. Biol. *4*, 303–321.

Shatz, C. J., and Stryker, M. P. (1988). Prenatal tetrodotoxin infusion blocks segregation of retinogeniculate afferents. Science *242*, 87–89.

Silva, A. J., Stevens, C. F., Tonegawa, S., and Wang, Y. (1992a). Deficient hippocampal long-term potentiation in α-calcium-calmodulin

kinase II mutant mice. Science *257*, 201–206.

Silva, A. J., Paylor, R., Wehner, J. M., and Tonegawa, S. (1992b). Impaired spatial learning in α-calcium-calmodulin kinase II mutant mice. Science *257*, 206–211.

Staubli, U., and Lynch, G. (1990). Stable depression of potentiated synaptic responses in the hippocampus with 1–5 Hz stimulation. Brain Res. *513*, 113–118.

Stevens, C. F., Tonegawa, S., and Wang, Y. (1994). The role of calcium-calmodulin kinase II in three forms of synaptic plasticity. Curr. Biol. *4*, 687–693.

Stevens, C. F., and Wang, Y. (1994). Changes in reliability of synaptic function as a mechanism for plasticity. Nature *371*, 704–707.

Stryker, M. P., and Harris, W. A. (1986). Binocular impulse blockade prevents the formation of ocular dominance columns in cat visual cortex. J. Neurosci. *6*, 2117–2133.

Sunyer, T., and Sahyoun, N. (1990). Sequence analysis and DNA–protein interactions within the 5′ flanking region of the Ca^{2+}/calmodulin-dependent protein kinase II α-subunit gene. Proc. Natl. Acad. Sci. USA *87*, 278–282.

Tan, S.-E., Wenthold, R. J., and Soderling, T. R. (1994). Phosphorylation of AMPA-type glutamate receptors by calcium/calmodulin-dependent protein kinase II and protein kinase C in cultured hippocampal neurons. J. Neurosci. *14*, 1123–1129.

Thiel, G., Czernik, A. J., Gorelick, F., Nairn, A. C., and Greengard, P. (1988). Ca^{2+}/calmodulin-dependent protein kinase II: identification of threonine-286 as the autophosphorylation site in the α subunit associated with the generation of Ca^{2+}-independent activity. Proc. Natl. Acad. Sci. USA *85*, 6337–6341.

Waldmann, R., Hanson, P. I., and Schulman, H. (1990). Multifunctional Ca^{2+}/calmodulin-dependent protein kinase made Ca^{2+} independent for functional studies. Biochemistry *29*, 1679–1684.